Potential Signaling Partners of Sry-Related HMG Box-C (SOXC) Transcription Factors in Renal Development

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Masters of Science

Department of Biomedical Sciences

Faculty of Veterinary Medicine

University of Prince Edward Island

Junzhuo Huang

Charlottetown, PEI

June, 2013

CONDITIONS FOR THE USE OF THE THESIS

PERMISSION TO USE GRADUATE THESIS

Title of Thesis: "Potential Signaling Partners of the Sry-Related HMG Box-C (SOXC) Transcription Factors in Renal Development"

Name of Author: Junzhuo Huang

Department: Biomedical Sciences

Degree: Master of Science **Year:** 2013

Name of Supervisor(s): Drs. Sunny Hartwig and Glenda Wright

In presenting this thesis in partial fulfilment of the requirements for a graduate degree from the University of Prince Edward Island, the author has agreed that the Robertson Library, University of Prince Edward Island, may make this thesis freely available for inspection and gives permission to add an electronic version of the thesis to the Digital Repository at the University of Prince Edward Island. Moreover the author further agrees that permission for extensive copying of this thesis for scholarly purposes may be granted by the professor or professors who supervised the author's thesis work, or, in their absence, by the Chair of the Department or the Dean of the Faculty in which the author's thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without the author's written permission. It is also understood that due recognition shall be given to the author and to the University of Prince Edward Island in any scholarly use which may be made of any material in the author's thesis.

Signature: _____

Address: Departmert of Biomedical Sciences Atlantic Veterinary College University of Prince Edward Island University Avenue, 550 Charlottetown, PE C1A 4P3
CANADA.

Date: _____

University of Prince Edward Island
Faculty of Veterinary Medicine
Charlottetown

CERTIFICATION OF THESIS WORK

We, the undersigned, certify that Junzhuo Huang, candidate for the degree of Master of Science, has presented his thesis with the following title:

Potential Signaling Partners of the Sry-Related HMG Box-C (SOXC) Transcription Factors in Renal Development

that the thesis is acceptable in form and content, and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate through an oral examination held on June 10, 2013.

Examiners:

Dr. Luis Bate: _____

Dr. Sunny Hartwig: _____

Dr. Sandra McConkey: _____

Dr. Collins Kamunde: _____

Dr. Karen Samis: _____

Date: _____

TABLE OF CONTENTS

ABSTRACT

SOXC transcription factors are key transcriptional regulators that have been shown to control self-renewal and differentiation in tissues during development *in vivo*, but have not been studied in the mammalian kidney. Our lab has previously demonstrated that all three members of the *SoxC* genes family, namely, *Sox4, Sox11* and *Sox12,* are co-expressed with the *Wilms' Tumor Suppressor-1 (Wt1)* gene, which is known to be essential in nephron progenitor cells during the early stages of kidney development. We have also demonstrated an essential role for at least one member of the SoxC subfamily, *Sox4,* during renal development *in vivo.* As a first step towards identifying potential SOXC co-factors during embryonic kidney development, I compiled a comprehensive list of all proteins shown to interact with SOX family members to regulate transcription *in vitro.* The list was then expanded to include highly homologous members of the same family or subfamily. Next, reverse transcription (RT)-PCR and quantitative real-time (q) PCR was performed for candidate partner genes at multiple time points of renal development. Candidate SOXC partner genes that exhibited strong renal developmental expression patterns included members of the *Dlx, Pou, Pax, Ube,* and *Med* families of transcription factors. Results from this work lay the foundation to elucidate the molecular mechanisms by which SOXC proteins regulate transcription during renal development *in vivo.* Future experiments will include cross-linking protein interaction analyses and pull-down assays to identify protein-protein interactions *in vitro*.

ACKNOWLEDGEMENTS

Firstly, I'd like to express the deepest appreciation to my supervisors, Drs. Sunny Hartwig and Glenda Wright. I have been blessed to have had such encouragement and leadership from my supervisors who brought me into the field of science and taught me how to grow as a scientist. You both taught me many valuable things, given me many opportunities, and I will never forget the faith and respect you have shown me. I'd like to thank my supervisory committee for taking the time to advise and support me. The completion of my thesis would also have not been possible without the expertise of several other individuals. In this respect, I would like to thank Monique Saleh, Joy Knight and Ashley Patriquen for all the time and patience spent on me, and providing invaluable assistance and instruction.

One of the biggest sources of support I have had has been from my parents. They have been a source of encouragement, love, shelter, and nourishment throughout this process.

I'd like to express my appreciation for my beautiful fiancée, Jenny, for her love and support, and for helping me keep a positive outlook. This would have been more strenuous without her support.

Final thanks go out to the Department of Biomedical Sciences for their contribution to my graduate student stipend.

DEDICATION

For

Dr. Sunny Hartwig who encouraged me to enter the forest of sciences

and

My parents and fiancée who inspired me along my journey within.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

^{O}C	Degrees Celsius
µl	Microliter
BMP	Bone morphogenetic protein
bp	Base pair
CAKUT	Congenital anomalies of the kidney and urinary tract anatomy
cDNA	Complementary DNA
CO_2	Carbon dioxide
COL2A	Collagen, type II, alpha
c-RET	Proto-oncogene c-RET
Ct	The cycle number at the threshold level of log-based fluorescence
CTBP	C-terminal binding protein
DDS	Denys-Drash syndrome
DLX	Distal-less homeobox
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
E	Primer efficiency
EGR	Early growth response gene
EP	E1A binding protein
FGF	Fibroblast growth factor enhancer
FGF	Fibroblast growth factor
FS	Frasier syndrome
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GDNF	Glial cell derived neurotrophic factor
GFP	Green fluorescent protein
H_2O	Water
HDAC	Histone deacetylase
HHEX	Hematopoietically expressed homeobox
HMG	High mobility group
HnRPK	Heterogeneous nuclear ribonucleoprotein K
HOX	Homeobox

HPRT	Hypoxanthine phosphoribosyltransferase
LEF	Lymphoid enhancer binding factor
M	Slope of standard curve
MED	Mediator complex
MEF	Myocyte enhancer factor
MET	Mesenchymal-to-epithelial transition
min	Minutes
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MM	Metanephric mesenchyme
mRNA	Messenger RNA
ng	Nanogram
nM	Nanomolar
NPC	Nephron progenitor cells
OCT	Octamer-binding protein
OLIG	Oligodendrocyte lineage transcription factor
OTX	Orthodenticle homeobox
P53	Tumor protein 53
PAX	Paired box gene
PBS	Phosphate buffered saline
PGC	Peroxisome proliferator-activated receptor gamma, coactivator
PGK	Phosphoglycerate kinase
POU	POU class
R^2	Correlation coefficient
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
RUNX	Runt-related transcription factor
SALL	Sal-like gene
sec	Seconds
SF	Steroidogenic
SF	Splicing factor
SIX	Sine-oculis homeobox
SOX	Sry-related high mobility group box

SRY	Sex-determining region Y
TCF	T-cell factor
TIP	K lysine acetyltransferase
TR	Thyroid hormone receptor
UB	Ureteric bud
UBE	Ubiquitin-conjugating enzyme
UNC	Nematode protein
UV	Ultraviolet
WAGR	Wilms tumor, aniridia, genitourinary anomalies, and mental retardation
WNT	Wingless-type MMTV integration site family
WT1	Wilm's tumour suppressor-1

GENE AND PROTEIN NOMENCLATURE

Gene and protein symbol conventions ("*Sry-related high mobility box-4*" gene)

Species	**Gene**	**Protein**
Homo sapiens	*SOX4*	SOX4
Mus musculus	*Sox4*	SOX4

1. INTRODUCTION

1.1 Kidneys

The kidneys are major organs of the urinary system. The vertebrate urinary system consists of two kidneys, two ureters, the bladder and the urethra. The urinary system performs essential functions to regulate both the volume and composition of body fluids as well as the elimination of metabolic waste products (Davidson et al., 2008; Little and McMahon, 2012).

1.1.1 Kidney structure and functions

The kidney is composed of a capsule, cortex and medulla. The renal capsule is a fibrous outer layer, the cortex is the peripheral layer and the medulla is the inner layer. Kidney shape differs among species. The human kidney has a typical multilobar form. The medulla of the human kidney contains multiple pyramidal structures. The apex or papilla of each pyramid is directed toward the renal pelvis. A medullary pyramid together with overlying cortex forms a renal lobe (Davidson et al., 2008) (Fig.1). Common laboratory animals such as rats and mice, as well as dogs, cats, horses, sheep

and goats have unilobar (unipapillary) kidneys, with the cortex and medulla fused into a single renal papilla (Eurell and Frappier, 2006). In all forms of kidney, urine drains from the papilla into the renal pelvis and from the renal pelvis to the bladder via the ureter. The functional units of the kidney are nephrons that are found within the medulla and cortex. A human kidney is comprised 200,000 to 1.8 million nephrons (Little and McMahon, 2012). The nephron is composed of a renal corpuscle composed of a glomerulus enclosed in a Bowman's capsule, proximal convoluted tubule, loop of Henle, and distal convoluted tubule. At the proximal end of the nephron, blood is filtered through the glomerular filtration barrier and the filtrate passes into the tubular nephron, which modifies the filtrate before delivering the urine to the collecting duct for disposal into the renal pelvis (Davidson et al., 2008).

The kidney participates in elimination of body waste, regulation of water and electrolyte balance, regulation of arterial blood pressure, regulation of acid-base balance, regulation of erythropoiesis, regulation of vitamin D synthesis and regulation of glucose synthesis (Guyton and Hall, 2006).

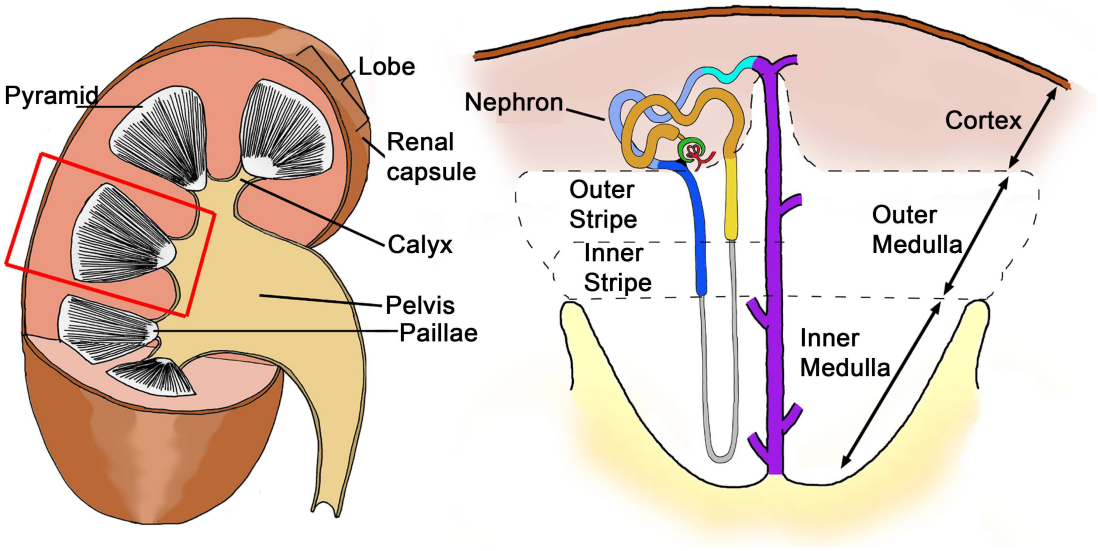


Figure 1. Structure of the human kidney (Modified from Davidson et al., 2008)

1.2 Kidney development

Development of the mammalian kidney is distinct from that of most other organs, proceeding through the formation of transitory kidney systems including the pronephros and mesonephros before forming the permanent functional kidney, the metanephros (Gilbert, 2010). The kidneys develop in a cranial to caudal direction within the intermediate mesoderm located between the paraxial mesoderm and lateral plate mesoderm in association with the nephric ducts (Chai et al., 2013). The first kidney to develop is the pronephros. The pronephros is not a functional kidney and quickly degenerates. The mesonephros begins development caudal to the pronephros. The mesonephros consists of glomeruli and mesonephric tubules that open into the mesonephric duct, a continuation of the nephric duct. The mesonephros also degenerates but tubules and ducts have derivatives in adult animals (Dressler, 2009). The metanephros (permanent kidneys) begins to form from the ureteric bud (UB), an outgrowth from the Wolffian duct, and the metanephric mesenchyme (MM). The metanephric kidneys are functional in the fetus (Chai et al., 2013).

1.2.1 Mouse metanephric kidney development

The development of kidneys in the mouse begins with the formation of the nephric duct (mesonephric/Wolffian duct) from the intermediate mesoderm and grows

caudally (Davidson et al., 2008) (Fig.2). The formation of mesonephric nephrons is induced by the Wolffian duct in the adjacent intermediate mesoderm (nephrogenic cord). The development of mouse metanephros begins at the caudal end of the nephric duct and nephrogenic cord.

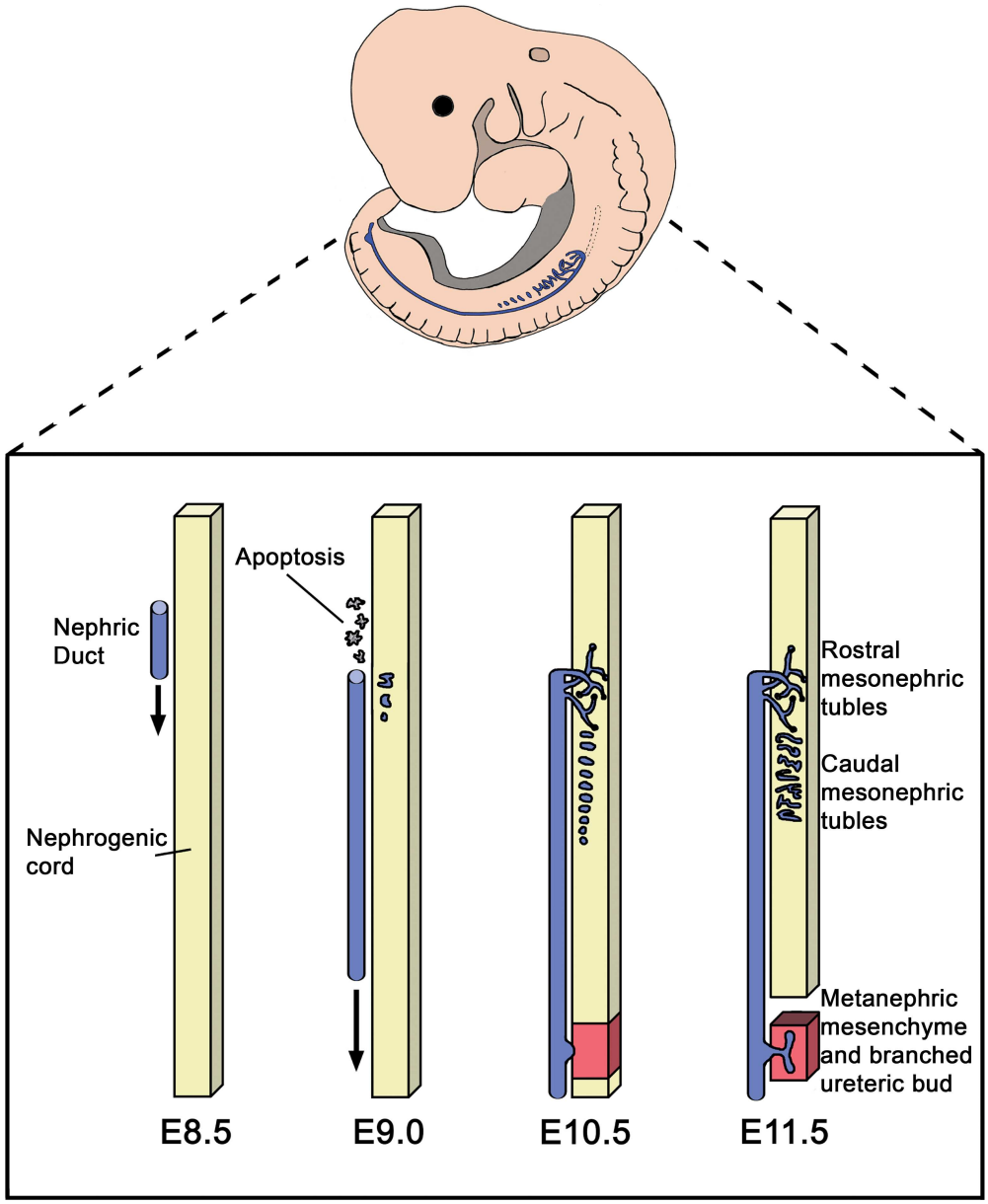


Figure 2. Overview of mouse kidney development at embryonic days 8.5, 9.0, 10.5 and 11.5 (E8.5, E9.0, E10.5 and E11.5 respectively) (Modified from Davidson et al., 2008).

1.2.2 Pronephros

The pronephros is the most primitive kidney. It is present only in the embryonic stage and is non-functional (Wessely and Tran, 2011). In the mouse, the pronephros is hardly detectable while mesonephric tubules are well developed (Dressler, 2009). The mouse pronephros develops from the intermediate mesoderm at embryonic day (E) 8.0 (Bouchard et al., 2000), at the level of presumptive somites 5-8. At that time, precursors of the pronephric duct separate away from the intermediate mesoderm in this region to form a short, caudally growing longitudinal rod of cells. Cells of the longitudinal rod undergo a mesenchymal-to-epithelial transition (MET) to generate the epithelial lined pronephric duct (Davidson et al., 2008) (Fig. 2). After formation, the cranial portion of the nephric duct degenerates by apoptosis. The duct persists in the more caudal part of the duct persists in the formation of the mesonephros and metanephros (Pole et al., 2002; Pietila and Vainio, 2005). In addition, some genes in the persisted duct are important for the subsequent development of the mesonephros and metanephros (Chai et al., 2013).

1.2.3 Mesonephros

The mesonephros forms in all vertebrates but degenerates in higher vertebrates such as mice and humans (Wessely and Tran, 2011). The mouse mesonephros develops along the caudal portion of nephric duct as the pronephros degenetrates at E10 (Dressler, 2009; Rumballe et al., 2010). The mouse mesonephros is composed of approximately 18

pairs of tubules, which extend from the somite level 10 to 17. The mesonephros is divided into distinct cranial and caudal sets of tubules (Sainio, 2003) (Fig. 2). The cranial set develops rudimentary branched tubules that join with the nephric duct at 4–6 sites (Sainio et al., 1997). Since the cranial mesonephros keeps contact with the Wolffian duct during the kidney development, it is believed that the cranial set of mesonephros is formed from the Wolffian duct (Joseph et al., 2009). In contrast, the caudal set of tubules make up the bulk of the mesonephros, and they are composed of primitive unbranched tubules that do not connect to the Wolffian duct. They are believed to be derived from the nephrogenic cord (Joseph et al., 2009). These unbranched tubules first appear as condensations of nephrogenic cord cells at the somites level 8-9 at E9 (Vetter and Gibley, 1966; Rumballe et al., 2010). These condensed cells undergo a MET to form a renal vesicle and then elongate into an S-shaped body (Dressler, 2009). At E14.5, the mesonephros begins to degenerate and within 24 hours, almost all of the tubules undergo apoptosis and disappear following in a caudal to cranial direction (Sainio et al., 1997; Little and McMahon, 2012). Eventually all glomeruli degenerate while the mesonephros disappears. However, some of the tubules persist in both sexes. In the male, the cranial tubules become the efferent ductules of the testis. In the female, the mesonephric tubules associated with the cranial and caudal part of the duct persist as residual structures in the mesosalpinx that is a part of the abdominal cavity from the ovary to the level of the uterine tube (Eurell and Frappier, 2006).

1.2.4 Metanephros

The metanephros, the permanent kidney, begins to develop when an outgrowth of the Wolffian duct grows into the surrounding metanephric mesenchyme (Fig. 2). This outgrowth is termed the ureteric bud. The ureteric bud epithelium invades the metanephric mesenchyme and begins to undergo branching morphogenesis (Chai et al., 2013). Some mesenchymal cells condense around the tips of newly formed branches to form nephron progenitor cells (NPC). The NPCs begin to undergo the process of MET under the inductive signal from the ureteric bud tips (Fig. 3). At the same time, other loose mesenchymal cells generate the interstitial stroma (Davidson et al., 2008). NPCs first form a polarized renal vesicle with one end of the vesicle retaining in contact with the ureteric bud epithelium. Then, a single cleft forms in the renal vesicle to generate a comma shaped body. A second cleft generates the S-shaped body. At this point, the S-shaped body has already patterned along a proximal-distal axis (Dressler, 2006). The S-shaped body is organized into three components: the distal, medial and proximal segments (Georgas et al., 2009). The distal end of the S-shaped body fuses with the ureteric bud epithelium to form a single, continuous epithelial tubule. At the proximal end, as endothelial cells invade the more proximal cleft, the S-shaped body forms the Bowman's capsule around the glomerular tuft (Quaggin and Kreidberg, 2008). Signaling interactions between mesenchymal derived epithelial cells of Bowman's capsule and endothelial cells lead to the formation of the glomerular basement membrane (GBM),

which is a highly specialized matrix that forms the scaffold for the filtration barrier (Chai et al., 2013). At the most proximal end are the precursor cells of the podocytes (Dressler, 2009).

The renal corpuscle containing the glomerulus is the most proximal part of the nephron, and it is an intricate structure, which includes a filtration barrier (Fig. 4). The glomerulus is a highly developed vascular bed that functions in filtration (Quaggin and Kreidberg, 2008). A mature glomerulus is composed of two distinct types of cells; endothelial cells and mesangial cells. The capillary endothelial cells form a fenestrated capillary tuft, which can allow proteins and fluid to pass through the endothelial wall (Quaggin and Kreidberg, 2008; Dressler, 2009). Mesangial cells are able to provide structure and elasticity to the capillary tuft, secrete extracellular matrix, and act as specialized pericytes to dilate or constrict capillaries thereby regulating blood flow (Fogo and Kon, 2010). The GBM separates the endothelial and mesangial cells from the urinary space. The GBM is a specialized basement membrane that is covered by the visceral epithelial cells of Bowman's capsule, the podocytes, whose extending foot processes interdigitate to create filtration slits, and a unique type of membrane, the slit diaphragm (Dressler, 2006; Quaggin and Kreidberg, 2008). The slit diaphragm separates the GBM from the urinary space (Dressler, 2009) and is involved in maintaining normal renal function by regulating a specific pore size such that only molecules smaller than approximately 5000 daltons can traverse the filtration barrier and enter the urinary space

(Quaggin and Kreidberg, 2008; Fogo and Kon, 2010). Therefore, damage to the slit diaphragm will lead to renal diseases (Quaggin and Kreidberg, 2008). The urinary space is surrounded by the parietal epithelium of Bowman's capsule and exits into the proximal convoluted tubule (Dressler, 2009).

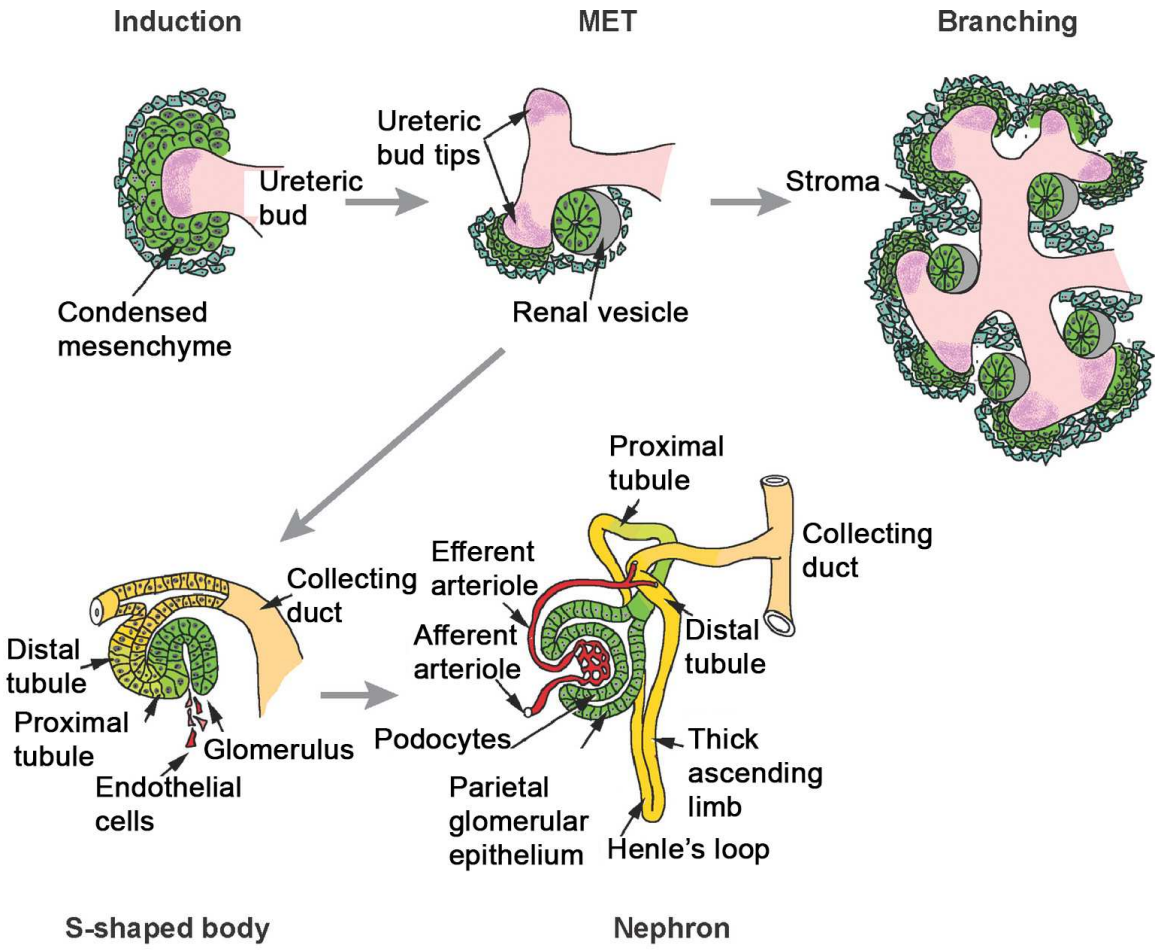


Figure 3. Sequential steps of nephrogenesis (Modified from Dressler, 2006).

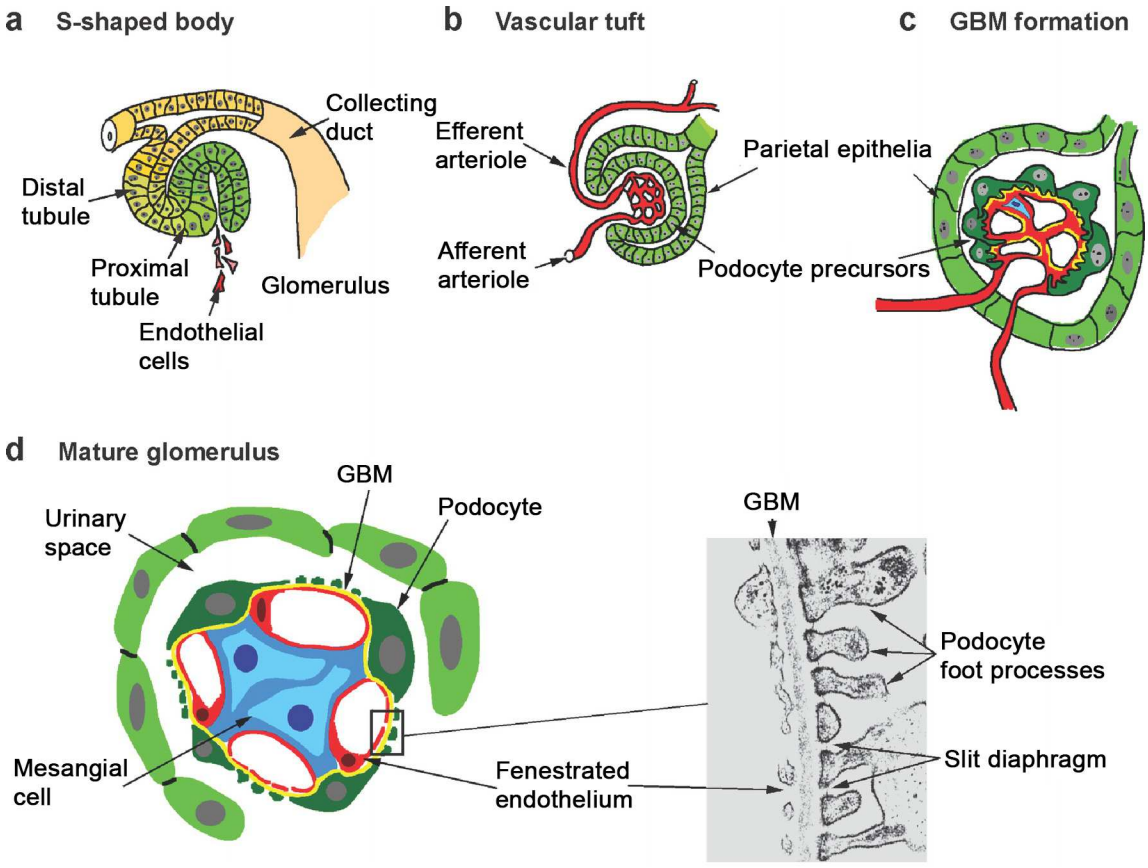


Figure 4. The sequential stages of glomerular development (Modified from Dressler, 2006).

1.2.5 Ureteric bud branching

In mammals, kidney development begins with an outgrowth at the posterior end of the Wolffian duct (at 5 weeks of gestation in the human and E10.5 in the mouse) (Chai et al., 2013). This outgrowth forms a simple epithelial tube called the ureteric bud which subsequently elongates and invades the surrounding metanephric mesenchyme. The part of the ureteric bud that invades the metanephric mesenchyme consists of a stalk and tip. The tip forms a swollen and rounder structure called the ampulla (Bridgewater and Rosenblum, 2009) (Fig. 5a). Under the inductive signal from the NPCs; the ampulla then divides to form two new ampullae, each of them will form a new branch (Fig. 5b). Tip-cell proliferation is thought to contribute to the formation of the next generation of tips and the lengthening of growing stalks (Bridgewater and Rosenblum, 2009). As organogenesis comes to completion, the UB branching slows down, presumably owing to negative feedback and finally forms the collecting ducts of the kidney. Abnormalities in the ureteric branch pattern can lead to renal malformations, including renal agenesis, renal dysplasia, and cystic abnormalities as well as alterations in nephron number (Costantini, 2010).

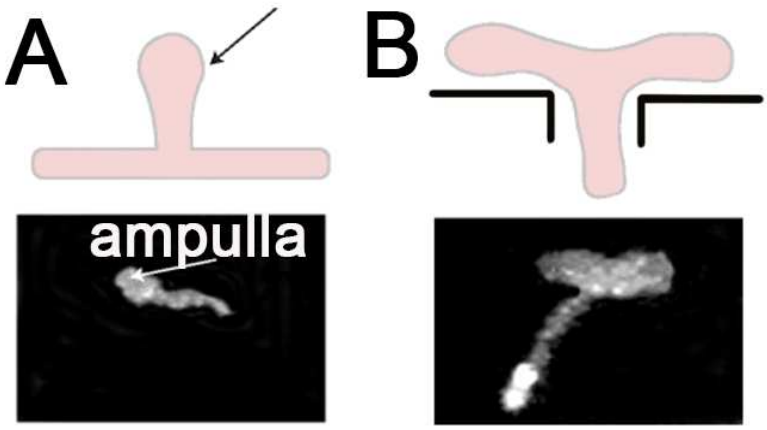


Figure 5. UB branching. (A–B) Schematic diagrams of branching events and images of cultured kidneys isolated from a Hoxb.7GFP transgenic mouse where green fluorescent protein (GFP) fluorescence marks the UB and its derivatives. (Modified from Bridgewater and Rosenblum, 2009)

1.3 Wilms' Tumor Suppressor 1(WT1)

Renal dysplasia is a common cause of young children's end-stage renal failure. It is characterized by abnormal cellular differentiation during renal development leading to tubular malformation. The congenital human kidney diseases Frasier Syndrome, Wilms tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) Syndrome, and Denys Drash Syndrome are all characterized by renal dysplasia, and are causally linked to mutations in the *Wilms' Tumour Suppressor-1 (WT1)* gene.

1.3.1 Kidney diseases

Congenital anomalies of the kidney and urinary tract (CAKUT): CAKUT is the most common cause of childhood end-stage renal disease, and comprise approximately 30% of all prenatally diagnosed malformations. CAKUT are variable in phenotype and can affect the kidney alone or involve the lower urinary tract. Congenital renal anomalies can be familial or sporadic familial, syndromic, or nonsyndromic. For the syndromic forms of congenital renal anomalies, genetic causes have been identified and have been investigated (Toka et al., 2010).

Denys-Drash Syndrome (DDS): Denys-Drash Syndrome is a relatively rare congenital childhood syndrome. DDS includes diffuse severe hypertension, progressive

glomerulopathy, a steroid-resistant nephrotic syndrome, and genital abnormalities. This syndrome rapidly progresses to end-stage renal disease before the age of five and bears a high risk of developing Wilms' tumor (Denys et al., 1967; Schumacher et al., 2006). The life-saving treatment of DDS is kidney transplantation and early bilateral nephrectomy before the development of Wilms' tumor (Mrowka and Schedl, 2000). Denys-Drash Syndrome is associated with *WT1* mutations of missense mutations in the zinc-finger region (Little et al., 2005)

Frasier Syndrome (FS): Frasier Syndrome is a slowly progressing nephrotic syndrome, which is attributable to focal segmental glomerulosclerosis. This is a high risk of development of end stage renal failure and complete male to female gender reversal but there is no development of Wilms' tumor (Frasier, 1964; Kitsiou-Tzeli et al., 2012). To date, *WT1* mutations have been found in many patients with Frasier syndrome. The *WT1* mutations are caused by mutations of the alternative splicing donor site in intron 9 (Zugor et al., 2006). In contrast to DDS, end-stage kidney disease develops more slowly and at a later stage in life (Mrowka and Schedl, 2000).

1.3.2 Wilms' Tumor

Wilms' tumor is the second most common intraabdominal cancer of childhood and the fifth most common pediatric malignancy. Approximately 6% of all pediatric cancers are Wilms' tumors and more than 95% of all kidney tumors in the pediatric age group are Wilms' tumors (Breslow et al., 1993; Pastore et al., 2006). In the United States there are approximately eight cases of Wilms' tumor per million children less than 20 years of age per year, with the total number of new cases being estimated at about 500 cases per year. Wilm's tumor occurs most commonly among children younger than five (Smith et al., 2010).

Wilms' tumor is an embryonic renal neoplasm that contains blastemal, stromal, and epithelial cells in varying proportions (Kim and Chung, 2006; Melchionda et al., 2013). There are various congenital anomalies known to associate with Wilms' tumor. Those anomalies include aniridia, hemihypertrophy, genitourinary tract anomalies such as cryptorchidism and hypospadias, DDS, and Beckwith-Wiedemann syndrome. These anomalies confer an increased risk of the development of Wilms' tumor (Kim and Chung, 2006). Wilm's tumor can be divided into three prognostic subgroups: low-risk tumors, the intermediate-risk group of tumors and the high-risk group tumors (Stewenius et al., 2007). Wilm's tumor also is believed to have a substantial heritable fraction since the tumor can be bilateral, and associated with congenital anomalies (Melchionda et al., 2013).

The formation of Wilms' tumor is complex, involving multiple genes and genetic events. Based on a number of studies, mutations in *WT1* are believed to be involved in the development of Wilms' tumor. One hundred percent of DDS patients were found to have *WT1* gene deletions or mutations (Rauscher, 1993; Kitsiou-Tzeli et al., 2012). The *WT1* gene encodes a transcription factor, which is critical to normal kidney and gonadal development. The exact role of *WT1* in tumor suppression remains unknown (Melchionda et al., 2013).

1.3.3 WT1

The human *WT1* gene is located on chromosome 11 and spans approximately 50 kb and includes 10 exons that generate a 3-kb mRNA (Call et al., 1990; Gessler et al., 1990; Chau and Hastie, 2012). There are four zinc finger motifs in the carboxyl-terminal portion of the WT1 DNA- binding domain, and they all share homology with the *early growth response gene 1(EGR1)* family. This suggests that the WT1 protein has a role as a transcription factor (Chau and Hastie, 2012). It has been shown that WT1 can bind to the promoter regions of downstream target genes. WT1 can either repress or activate the target gene (Sharma et al., 1992; Hartwig et al., 2010).

During embryogenesis, the expression pattern of *WT1* is highly complex (Mrowka and Schedl, 2000). For example, *Wt1* transcripts were found expressed at the

NPC during renal development in mice (Kreidberg, 2010). However, the expression of *Wt1* is weak during the condensation of the metanephric mesenchyme, and the expression of *Wt1* dramatically increases during the MET in the NPCs lineage (Mrowka and Schedl, 2000). *Wt1* expression is highest in the most proximal portion of the S-shaped body that flattens to form the podocytes. It has been reported that WT1 activity is essential for the transition of cells from mesenchymal and epithelial cell states (Chau and Hastie, 2012). Cells such as podocytes with the potential of epithelial to mesenchymal transition have been found to have *Wt1* expression (Grubb et al., 1994; Kreidberg, 2010). In the adult kidney, *Wt1* expression can be found in the podocytes, and the expression can be seen at a very lower level in parietal epithelial cells of Bowman's capsule (Chau and Hastie, 2012). In addition to the kidney, *Wt1 is* also expressed has an expression in the spleen (Herzer et al., 1999; Hartkamp et al., 2008), the mesothelium, and the genital ridges, which develop into testes or ovaries depending on the presence or absence of the Y chromosome (Nachtigal et al., 1998; Barrionuevo et al., 2012).

The complexity of *Wt1* expression suggests that WT1 is required at multiple stages during kidney development. In *Wt1* knockout mice, the outgrowth of ureteric bud is terminated and the metanephric blastema undergoes apoptosis (Kreidberg, 2010). Apoptosis was not terminated even when blastema from knockout mouse was recombined with ureteric buds from wild-type mouse during organ culture experiments.

These data suggest that *Wt1* has at least two functional roles during the initial part of the kidney development (Mrowka and Schedl, 2000). *Wt1* may be required for the inductive signaling, which induces the outgrowth of the ureteric bud, or it may be involved in either survival or the reception of the survival signal from the ureteric bud (Mrowka and Schedl, 2000; Kreidberg, 2010).

Therefore, identifying the direct target genes of WT1 will be essential for understanding the function of WT1 in developing organs including the developing kidney. From ChIP-Chip analysis, essential kidney development genes, such as *Pax2, Bmp7* and *Sall1* were identified as WT1 transcriptional targets, and all three members of the *Sry-related high mobility group (HMG) Box (Sox)-C* subfamily - *Sox4, Sox11* and *Sox12*- have also been identified as WT1 gene targets by ChIP-Chip (Hartwig et al., 2010).

1.4 SOX transcription factors

1.4.1 SOX family

SOX genes and their proteins belong to the high mobility group (HMG) superfamily, which consists of two subfamilies: the SOX family and the T-cell factor (TCF)/lymphoid enhancer binding factor (LEF-1) family (Laudet et al., 1993; Grosschedl et al., 1994; Soullier et al., 1999; Zhu et al., 2012). The male sex

determination gene *Sry* (sex-determining region Y) was the first-identified member of the *SOX* gene family (Koopman, 1999; Castillo and Sanchez-Cespedes, 2012). SOX proteins can bind specific DNA sequences. For example, SOX proteins bind the minor groove of DNA and bend it at an angle. This change of conformation may bring distal proteins on gene promoters closer together, allowing them to functionally interact. As a result, SOX proteins are regarded as developmental transcription factors (Kamachi et al., 2000; Kondoh and Kamachi, 2010).

SOX proteins can be identified based on a conserved motif within the HMG domain, RPMNAFMVW (Bowles et al., 2000; Kiefer, 2007). However, an intriguing exception is that the first characterized SOX protein, SRY protein, bears only RPMNAF. For proteins, a sequence motif is formed by the three dimensional arrangement of the amino acids and it has biological significance. Based on the phylogenic analysis of their HMG domains, *SOX* genes are separated into eight groups, A-H, including *(SOXA: Sry), (SOXB: SOX1, SOX2, SOX3, SOX14, SOX21), (SOXC: SOX4, SOX11, SOX12), (SOXD: SOX5, SOX6, SOX13), (SOXE: SOX8, SOX9, SOX10), (SOXF: SOX7, SOX17, SOX18), (SOXG: SOX15)* and *(SOXH: SOX30)* (Bowles et al., 2000; Kondoh and Kamachi, 2010).

The functions of SOX proteins include regulation, specification, and differentiation of many cell types, such as chondrocytes and the Sertoli cells of the developing testes. Proteins resulting from genes within the same group share functional roles and can counteract the functions of other groups. In addition, the same protein can

mediate different stages of development in one cell type or more. One possible answer to the question of why several *SOX* genes exist within each group is that they may provide a safety mechanism (Kiefer, 2007). If one SOX protein encoded by one *SOX* gene is malfunctioning, there would be always another protein within the same group to help instead. Kidney development is regulated by signal pathways at a molecular level such as the SOX transcription factors (proteins). These perform a variety of crucial roles in vertebrate development by activating or repressing specific target genes through interaction with different proteins (Uwanogho et al., 1995; Collignon et al., 1996; Kreidberg, 2010). It has been proposed that SOX proteins paired with protein partners have a significant role in the regulation of the embryonic development and the determinations of the fates of cell (Kamachi et al., 2000; Kondoh and Kamachi, 2010).

SOX genes have been found in all bilaterian metazoans, which is evidence that a full complement of the major groups of the *SOX* gene family was already established early in the evolution of the Bilateria (Bowles et al., 2000; Castillo and Sanchez-Cespedes, 2012). However, there is a dramatic difference in the number of *SOX* genes between the protostomes and the deuterostomes. Model protostomes such as the *Caenorhabditis elegans worm (*nematode) have few *SOX* genes, with numbers ranging between five and eight, while deuterostomes, such as mammals, have as many as 20 *SOX* genes which can then be separated into the above groups and further into subgroups (Bowles et al., 2000; Castillo and Sanchez-Cespedes, 2012). For example, the five *SOXB* genes found in deuterostomes can be further separated into *SOXB1* and *SOXB2*

subgroups (Bowles et al., 2000; Kiefer, 2007).

1.4.2 SOXC subfamily: SOX4, SOX11 and SOX12

Based on phylogenic study, the SOXC subfamily consists of three highly homologous proteins, SOX4, SOX11 and SOX12 (Dy et al., 2008). These proteins show similar DNA-binding properties and transcription potentials. All SOXC proteins are widely and dynamically expressed during mouse embryogenesis. For example, it has been shown that *Sox4* and *Sox11* genes regulate the late steps of neural development (Bergsland et al., 2006). In addition, *SOXC* genes can directly activate early genes, which can give cells general neuronal properties. This is necessary for cell type-specific differentiation. The *SOX11* gene plays a role in cardiogenesis, regulating outflow tract formation, development of multiple organs (lung, stomach, pancreas, spleen, eye and skeleton), and pan neuronal gene activation during neuronal maturation (Schilham et al., 1996; Hargrave et al., 1997; Kuhlbrodt et al., 1998a; Zhu et al., 2012). In conclusion, *SOX4* and *SOX11* genes have significant roles in developmental processes, but their molecular roles in many lineages and the roles of *SOX12* gene remain largely unknown.

A recent study has shown that all three members of the SOXC subfamily are expressed in nephron progenitor cells, ureteric bud tips and forming nephrons in developing mouse kidney (Huang et al., 2013). In addition, the conditional knock out of

Sox4 in NPC leads to the reduction of nephron numbers and malformation of podocytes during mouse renal development (Huang et al., 2013). These all suggest that SOXC transcription factors may play essential roles in mouse nephrogenesis.

1.5 Protein partners of SOXC transcription factors

It has been shown that SOX proteins often pair off with specific protein partners to regulate gene transcription (see Table 1 in Results). This mechanism allows SOX proteins to initiate a sharp transition in the set of regulated genes, probably either in accordance with the change of the partner factor or due to dynamism of the expression of the SOX (Kondoh and Kamachi, 2010).

1.5.1 SOX protein partners

Several SOX factors are expressed in more than one cell type, and they regulate different events in the same cell type. For instance, SOX9 is expressed in developing chondrocytes and also in Sertoli cells of developing testis. SOX18 is expressed in developing vascular endothelial cells and in mesenchymal cells subjacent to invaginating hair and feather follicles (Bernard and Harley, 2010). All SOX protein factors recognize a similar DNA-binding motif (A/TA/TCAA A/TG). Certain SOX factors are present in several different types of cells and those cells can express several

different kinds of SOX proteins. Therefore, the question arises, how does each SOX factor recognize its target gene?

Target gene specificity or regulation is achieved by binding with specific protein partners in order to direct gene expression for a particular cell type. Regulated genes have binding sites for both SOX proteins and their partner proteins on their enhancer sequences (Kondoh and Kamachi, 2010). Accumulated evidence shows that SOX proteins form multi-protein complexes at gene promoter or enhancer sites, and that these complexes enable stable DNA binding, *SOX* gene specificity and activation of *SOX* gene. Based on these studies, Kamachi et al. (2000) proposed a model suggesting that binding of the SOX transcription factor to its site on the gene enhancers is not sufficient to exert its regulatory function because SOX proteins, in most cases, bind to DNA with a dissociation constant of 10^{-8} - 10^{-9} M *in vitro*, which might not be stable enough to exert transactivation *in vivo*. On the other hand, abundant protein activators bind to DNA with a dissociation constant of 10^{-11} M *in vitro*. Because of this low DNA binding affinity of SOX protein, assistance of a co-DNA-binding partner factor might result in the achievement of stable binding and successful activation of the gene *in vivo*. This model also sheds light on how valuable SOX-partner pairing is and how the pairing increases the strength and stability with which SOX transcription factors bind to their respective proteins (Kondoh and Kamachi, 2010). For example, SOX2 is important for lens development, and it can bind to *δ-crystallin* gene minimal enhancer, DC5, which directs lens-specific gene expression. This binding requires a partner factor called δEF3

(Kamachi et al., 2000). SOX9 is important for cartilage and genital ridge formation by activation of its target gene *Col2a1*, which encodes type II collagen. This binding requires a partner protein COL2C2 (Kondoh and Kamachi, 2010).

In an *in vivo* situation where DNA is in a chromatin structure, the HMG domain of SOX is not sufficient to form a stable protein–DNA complex at a SOX site, although its DNA binding is demonstrable *in vitro* (Kondoh and Kamachi, 2010) (Fig. 6a). A SOX protein forms a stable complex with the target DNA only in the presence of a partner factor, which interacts with the SOX protein and binds to a nearby DNA site. Transcriptional activation or repression by a SOX protein is achieved only under this condition (Fig. 6b). The partner factor at a SOX-binding site of an enhancer is determined by two conditions: first, the availability of the binding site for a partner factor in a proper location and orientation; and second, the availability of the partner factor in the cell (Fig. 6c).

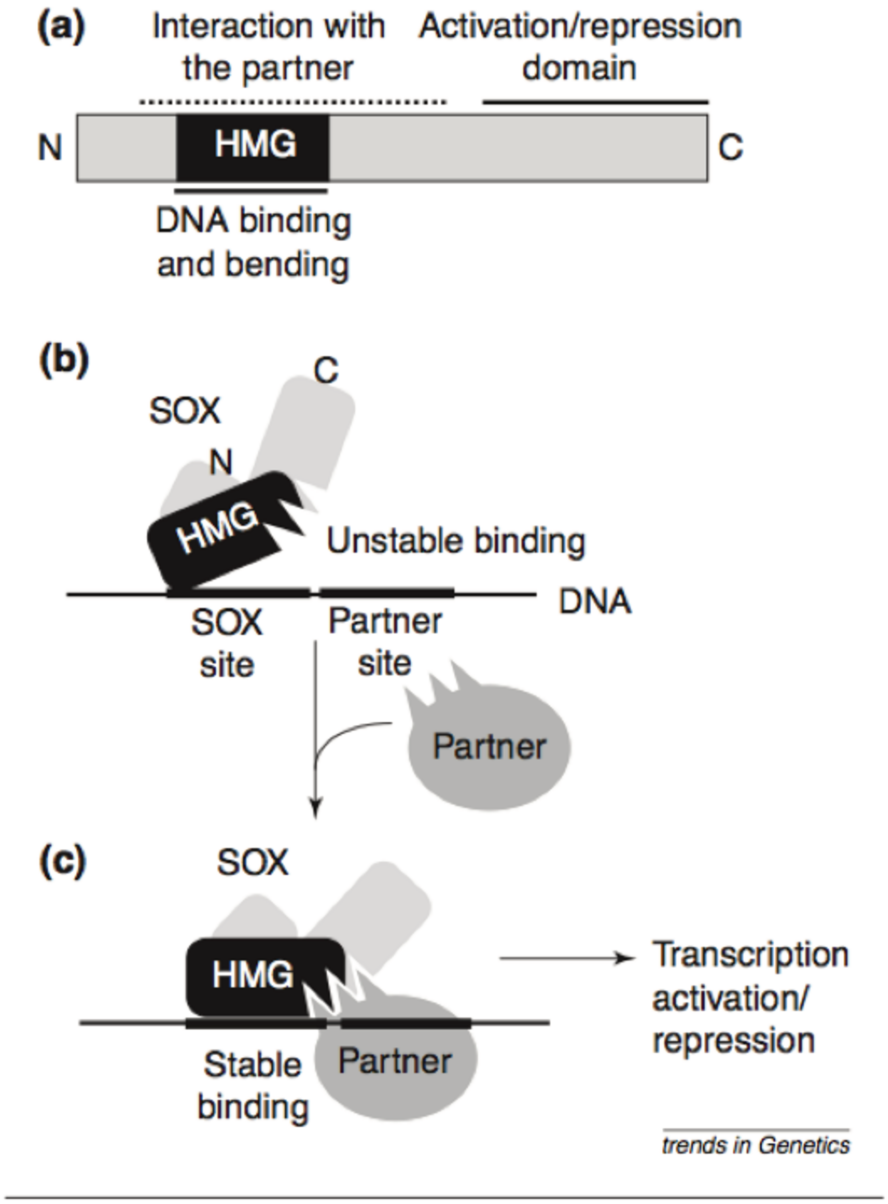


Figure 6. The model of gene regulation by SOX transcription factors synergizing with their partners (Modified from Kamachi et al., 2000). A SOX transcription factor (a) has three functional domains: an HMG domain to interact with DNA; an activation domain or a repression domain close to the C-terminus; and a region that include a part of the HMG domain to interact with the protein partner. When a SOX transcription factor binds to a target gene through its HMG domain alone (b), the binding is unstable. When the partner factor interacts with the SOX transcription factor and binds to the DNA site next to the SOX site (c), then the SOX protein will be stabilized on the DNA. A typical SOX transcription factor (SOX), HMG domain (HMG), C-terminus (C), N-terminus (N).

1.5.2 SOXC protein partners

SOXC proteins interact with protein partners to regulate gene expression. The three SOXC proteins have conserved, overlapping expression patterns and have similar molecular properties. These might therefore act in concert to fulfill essential roles *in vivo*. The SOX4 protein encoded by the *SOX4* gene can interact with syntenin (PDZ protein) in B cells, but how the genes are regulated is not yet known (Geijsen et al., 2001). SOX11 protein can interact with Brn-1 (a member of POU protein family) in glial cells, but how the genes are regulated is also not yet determined (Kuhlbrodt et al., 1998a). All SOX transcription factors can recognize a similar core DNA binding motif. Each of the SOX transcription factors can be expressed in a variety of cellular contexts, and a given cell type can co-express a number of SOX factors. The specificity of the interaction between a certain SOX transcription factor with the target gene is determined by the protein partners (Kondoh and Kamachi, 2010). Identifying which protein partners interact with SOXC transcription factors is the key to understanding the roles of *SoxC* genes in renal development since SOXC transcription factors need the specific protein partners to maintain the specificities during the renal development.

1.6 Research objectives

1.6.1 Rationale

SOX transcription factors play master regulatory roles in a multitude of developmental processes, including stemness, male differentiation, chondrogenesis, neurogenesis and gliogenesis, as well as development of the neural crest, spinal cord and heart (Bowles et al., 2000). However, their function in the developing kidney is largely undefined. All SOX proteins recognize a similar core DNA binding motif, and a given cell type can co-express a number of SOX factors. Despite weak DNA binding specificities, individual SOX proteins recognize different target genes. Target gene specificity is thought to be attributable in part, to the formation of multi-protein complexes comprised of a SOX protein and cell-specific cofactors at gene promoters or enhancers. To date, only a limited number of Sox protein partners have been identified. We have recently demonstrated that all three highly-related members of the *SoxC* subfamily are strongly expressed in nephron progenitor cells and their derivative nephrogenic structures during renal development. We have also demonstrated an essential role for *Sox4* during renal development *in vivo* (Huang et al., 2013). Identifying SOXC protein partners during kidney development will help us to comprehensively understand the SOXC regulatory network during the kidney development.

1.6.2 Hypotheses

In this study, the following hypotheses were tested:

1. There are highly homologous members of SOX protein partners.

2. Potential protein partners of SOXC transcription factors are highly expressed during mouse kidney development.

3. Potential protein partners of SOXC transcription factors share a similar mRNA expression pattern as SOXC subfamily.

2. MATERIALS AND METHODS

2.1 Tissue isolation

CD-1 embryonic and adult mice (*Mus musculus*) provided by the Atlantic Veterinary College, UPEI were used in this study. All experiments were carried out in accordance with the UPEI Animal Care Committee guidelines. Wild type mice were paired overnight. The detection of a vaginal plug the next morning staged the embryos as embryonic (E) 0.5 days old. Three litters each of E12.5, E13.5, E14.5, and E18.5 day embryos from 3 different female wild type mice and 3 each of postnatal (P) 7 day and P21 day (adult) mice were dissected and kidneys collected. Kidneys from different litters were separated in *RNA later*^{tm} on ice and stored in -80^{o}C until RNA isolation was performed.

2.2 RNA isolation and reverse transcription

Separated tissues were homogenized by a Rotor stator with β-mercaptoethanol. Tissue lysates were applied to RNA isolation by using an *Illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare Inc)*. All of the steps were carried out on ice following the manufacturer's instructions except that the total RNA was eluted with 200μl nuclease free H_2O *(GIBCO Inc)*. The steps in order are: 1) homogenization of kidney tissues

using the Rotor Stator, 2) cell lysis, 3) filtration of the lysate, 4) adjustment of RNA binding conditions, 5) binding of the RNA, 6) desalting of the silica membrane: This prepares favourable conditions for DNA digesting by DNases because salt removal makes the subsequent DNase digest much more effective, 7) digesting DNA: Since DNA and RNA bind to the silica membrane, DNases to thoroughly digest DNA, 8) washing and drying the silica membrane, 9) eluting highly pure RNA. The detailed protocol can be found in the Appendices.

The quality and concentration of total RNA were measured by using *NanoDrop ND-1000 spectrophotometer (Thermo Inc.)* and agarose gel electrophoresis. All samples had concentrations of 100- 300 ng/μl, a 260/280 ratio of 1.8-2.2, a 260/230 ratio above 1.8 and two clear bands of 28S and 18S RNA by gel electrophoresis.

The mRNA was reverse transcribed into cDNA using the *Quanta qScript supermix cDNA synthesis kit (Quanta Inc)*. A mixture of 4μl of 5x super mix, 1μg of RNA template and a total reaction volume of 20μl with nuclease free H_2O was incubated for 5 minutes at 25ºC followed by 30 minutes at 42 ºC and 5 minutes at 85ºC. cDNA was diluted 20x in nuclease free H_2O and stored at -40ºC for RT-PCR. After cDNA synthesis, cDNA purity was tested by agarose gel electrophoresis. To test the quality of the cDNA, the primer for the house keeping gene *Gapdh* and the primer for *Six2* were used. High quality cDNA should show the same bands width in each stage in the agarose gel analysis. The *Six2* gene is a marker of NPC and usually expressed in early embryonic development such as the E13.5 stage. It is expressed at lower levels in the E18.5 stages

Table 1. mRNA concentration (ng/μl) of four biological replicates of mouse kidney tissues at E13.5, E18.5 and P21 for RT-PCR.

	mRNA Concentration (ng/μl)		
Biological Replicate	E13.5	E18.5	P21
1	172.9	299.2	146.2
2	206.1	615.6	398.0
3	141.1	538.2	376.3
4	158.0	691.1	448.4

Table 2. mRNA concentration (ng/μl) of four biological replicates of mouse kidney tissues at E12.5, E14.5, E18.5, P7 and P21 for Real-time PCR.

	mRNA Concentration (ng/μl)				
Biological Replicate	E12.5	E14.5	E18.5	P7	P21
1	106.4	218.0	540.0	117.1	376.2
2	127.1	150.0	617.0	119.0	448.0
3	130.8	218.0	771.3	114.0	89.6

and minimally expressed in adults; therefore, the *Six2* bandwidth in high quality cDNA from E13.5 samples should be almost double compared to that in E18.5 stage samples (Fogelgren et al., 2009). In addition, the bandwidth in adults should be negligible (Fig.7).

The following RT-PCR and real-time PCR identified the expression level of each gene based on the cDNA template. Therefore, to have precise comparison of the expression level of each gene, the exact same amount of RNA should be added during the cDNA synthesis.

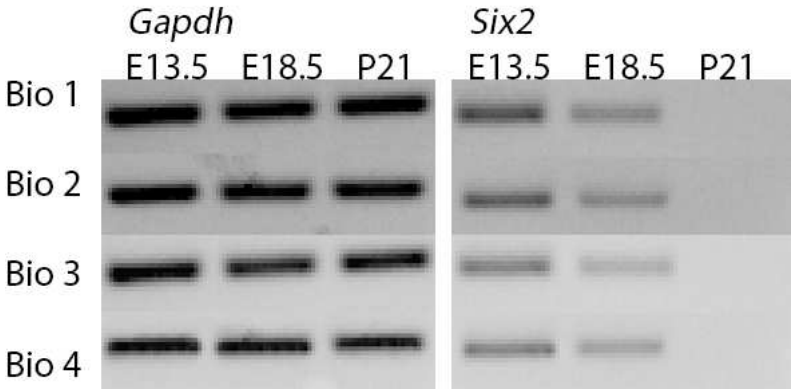


Figure 7. *Gapdh* and *Six2* gene expression on four biological replicates of mouse kidney cDNA at E13.5, E18.5 and P21. Biological replicate 1(Bio 1), Biological replicate 2(Bio 2), Biological replicate 3(Bio 3), Biological replicate 4(Bio 4).

2.3 Primer design and Reverse Transcription (RT)-PCR

I first sought to list all genes reported to interact with members of the *SOX* gene family. The list of genes was treated as potential SOXC partners. The NCBI PubMed database was chosen as the main resource used to search for previously published articles on the *SOX* gene family and their interactions with other proteins.

Primers were designed using *PrimerBank* (http://pga.mgh.harvard.edu/primerbank/), and selected primers were confirmed for sequence specificity by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/). Primer lengths of 18-24 base pair (bp) were chosen for both forward and reverse primers and the amplicon sizes of 100-200 bp were chosen to maximize amplification efficiency overlapping intron-exon boundaries. Primers were purchased from *Sigma Aldrich*.

RT-PCR was used to show whether specific genes were expressed in four biological replicates of cDNA obtained from murine kidneys at E13.5, E18.5 and P21. The PCR was carried out using *Econotaq MasteMix* (*Lucigen Inc*). PCR conditions were optimized to show specific bands. *Gapdh* and *Six2* were used as the positive controls. Agarose gel electrophoresis was conducted after RT-PCR. Ethidium bromide was used as the fluorescent marker. The gel electrophoresis images were analyzed by the image software *Gel Analyzer (Lazar software Inc)*. Every candidate partner's gel band pixel intensity was measured using this software. The fold change of pixel intensity at E13.5

compared to P21 was used as an indicator of developmental regulation to narrow down the list. The genes having fold changes above 1.45 were chosen for further research. One example is *Pou4f2*, which has a fold change of 114. There is no difference among E13.5, E18.5 and P21 at or below this fold change, for example, see *Smad 3* and *Med6* (Fig.16).

RT-PCR conditions were optimized to ensure that gene expression remained in the exponential stage. Optimized conditions also eliminates all non-specific bands. In cases where optimizing conditions could not be achieved, new primers were designed and used.

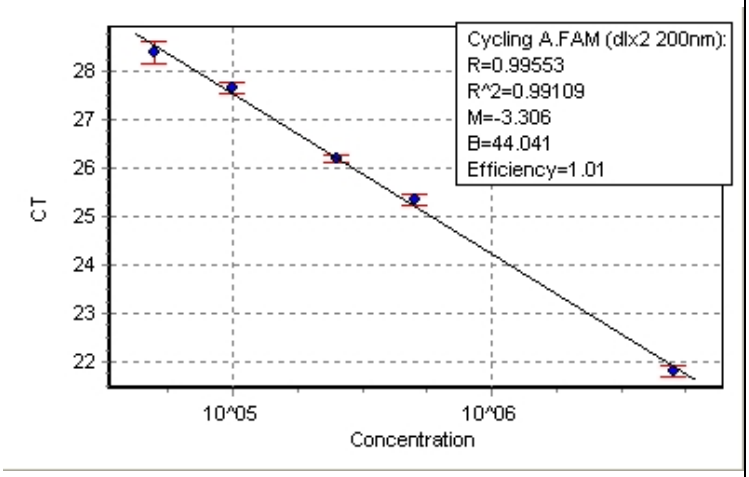
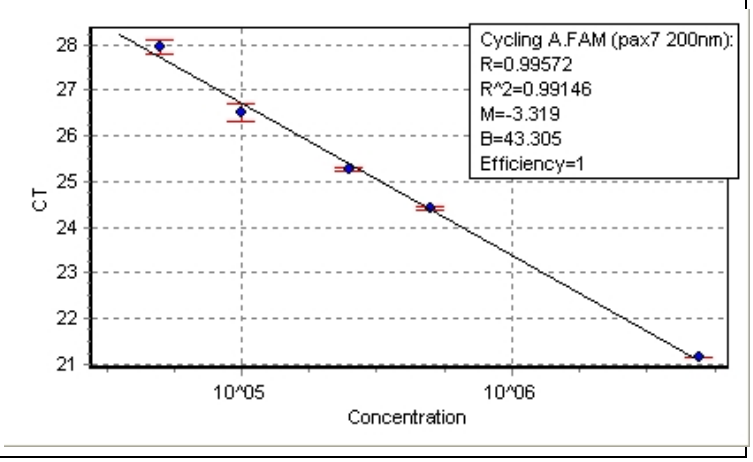
2.4 Real time PCR

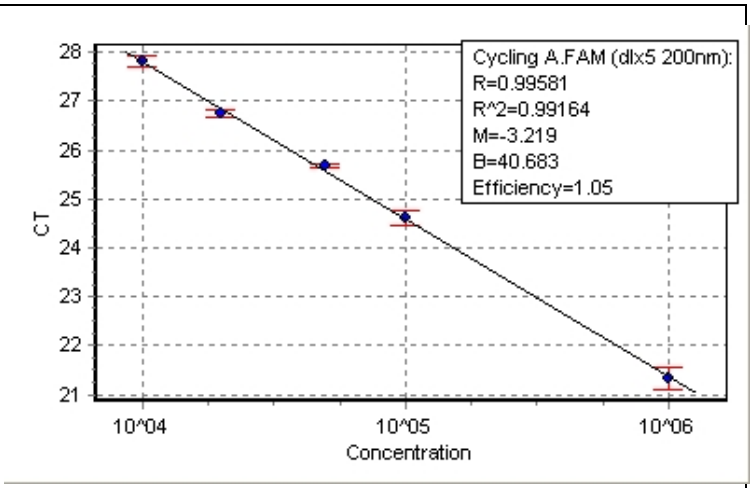
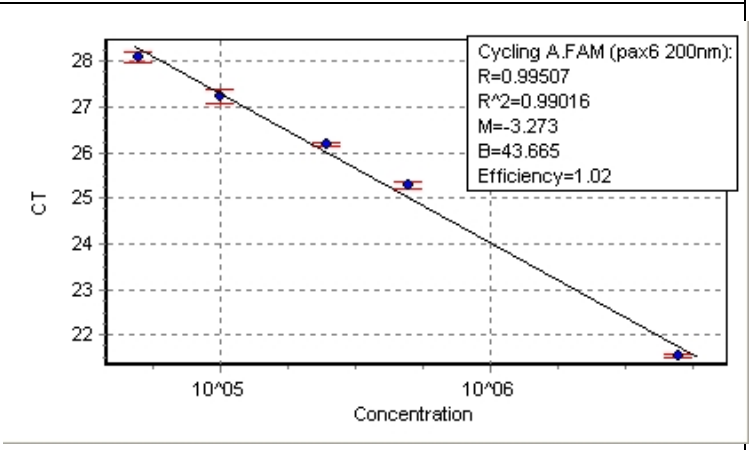
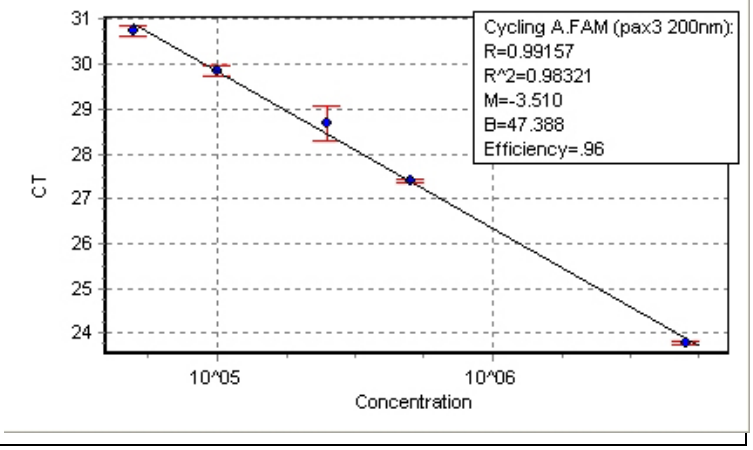
Real time PCR was conducted to quantitatively analyze the expression of the short-listed candidate SOXC protein partners. The efficiency of each primer from the short-listed candidate SOXC protein partners was tested on 3 biological replicates of cDNA generated from murine kidneys at E13.5. Each analysis was conducted in triplicate using the *Rotor-Gene 3000 detection system (Corbett Research Inc.)* with *PerfeCta^{TM} SYBR Green FastMix^{TM}, Low ROX^{TM} (Quanta Biosciences Inc.)* on 5µl of cDNA template using optimized primer concentrations. Each gene was tested on 5 dilutions including 1X, 10X, 20X, 50X and 100X. The primer concentrations and betaine were used to optimize reactions to obtain >0.99 R^2 and >0.95 efficiency. The *Rotor-Gene6 software (Qiagen Inc.)* was used to analyze the data and generate a

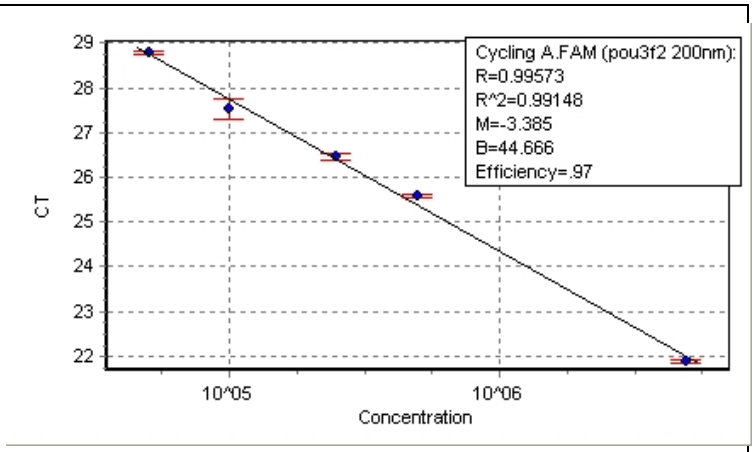
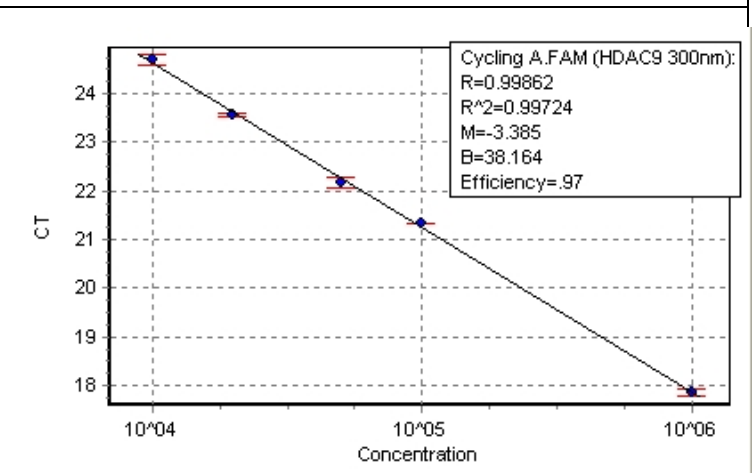
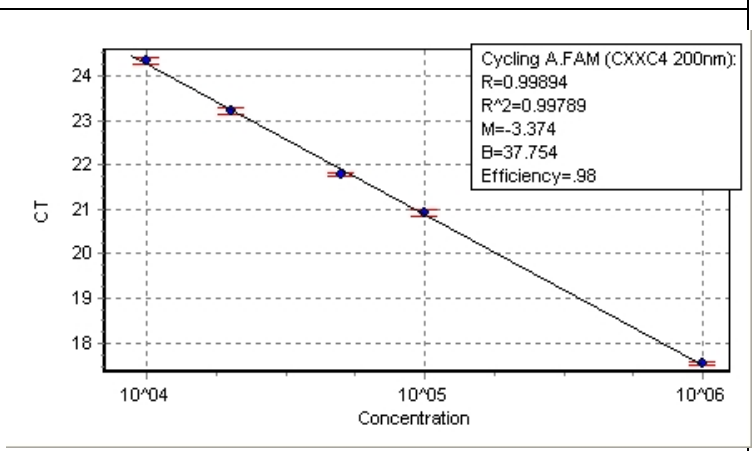
standard curve.

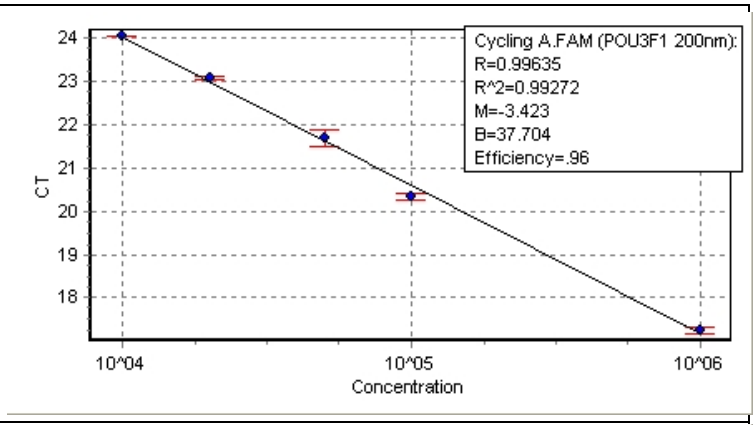
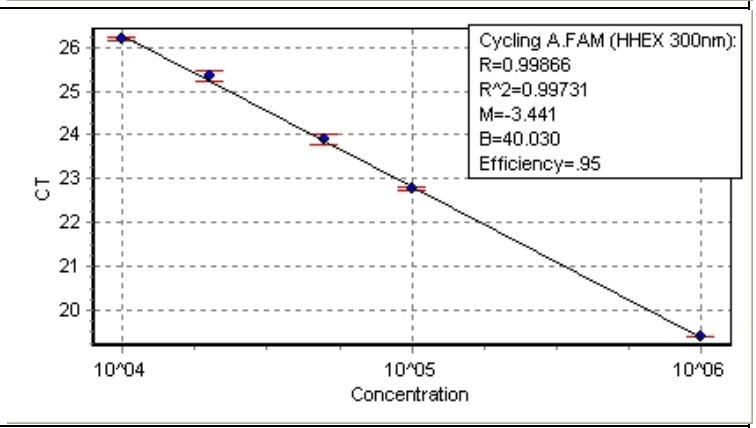
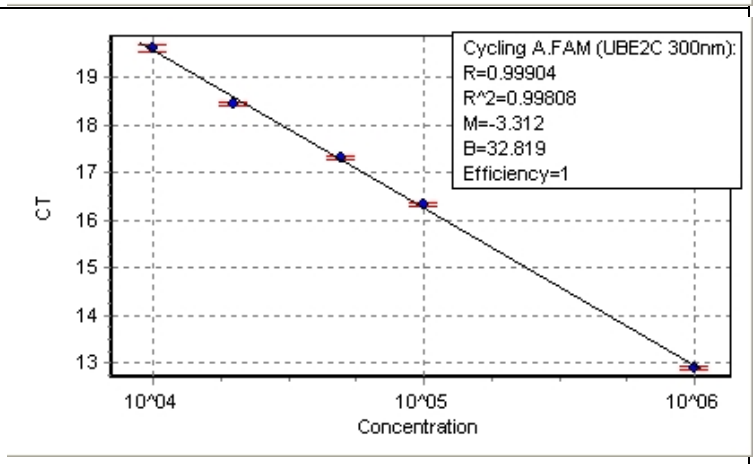
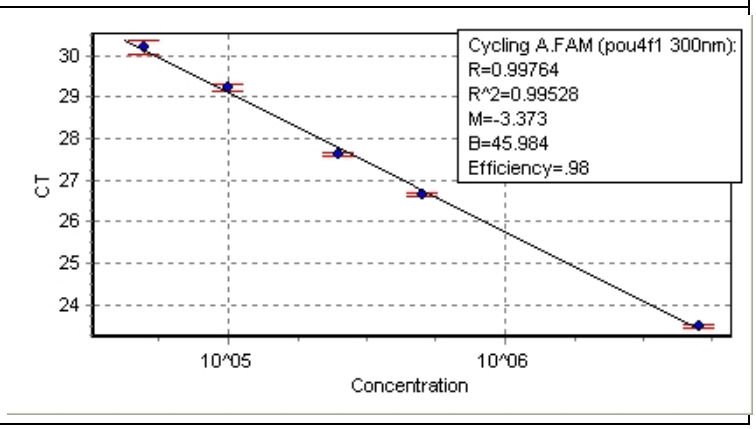
The primer efficiency must be over 0.95 to obtain a precise comparison. Therefore, the accuracy of the series dilution and cDNA template is critical. Vortex and pipetting were absolutely precise (Table 3).

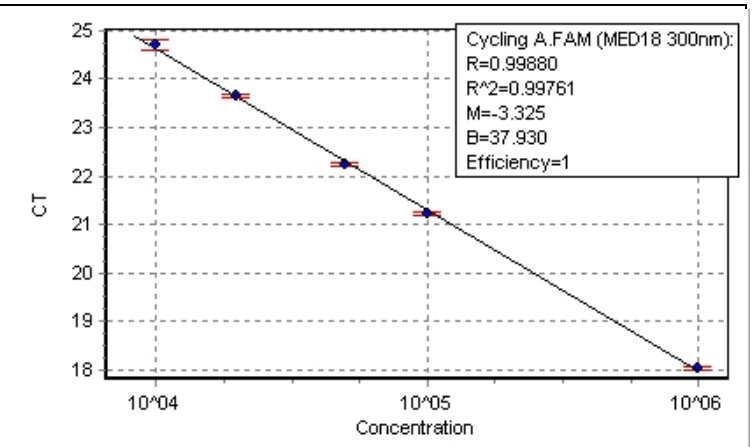
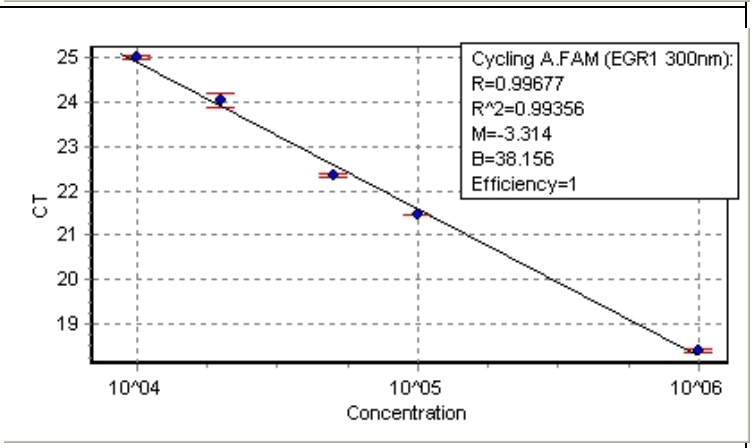
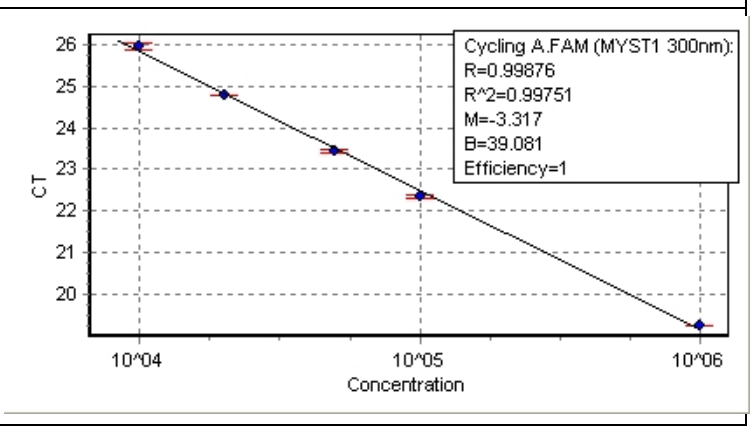
Table 3. Primer concentrations used to test short-listed potential protein partners of SoxC transcription factor; E (primer efficiency), R^2 (coefficient of determination in linear regression) value and standard curve (CT value over cDNA concentration) on each short-listed potential protein partner with standard error. R= Square root of correlation coefficient; M= Slope, B=intercept, according to the formula y=mx+b; n=3.

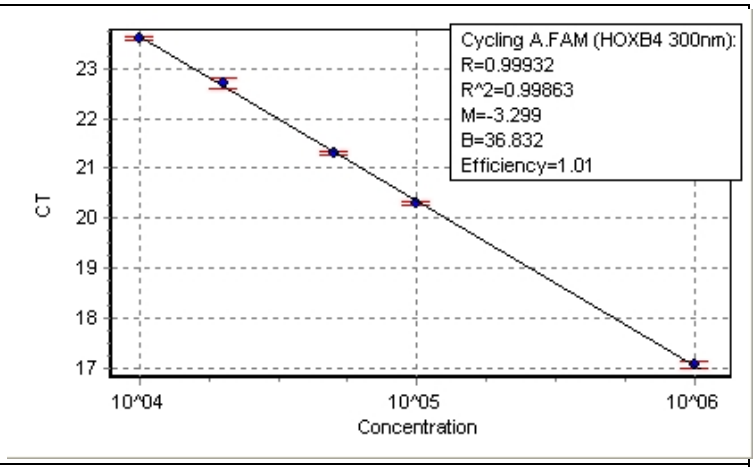
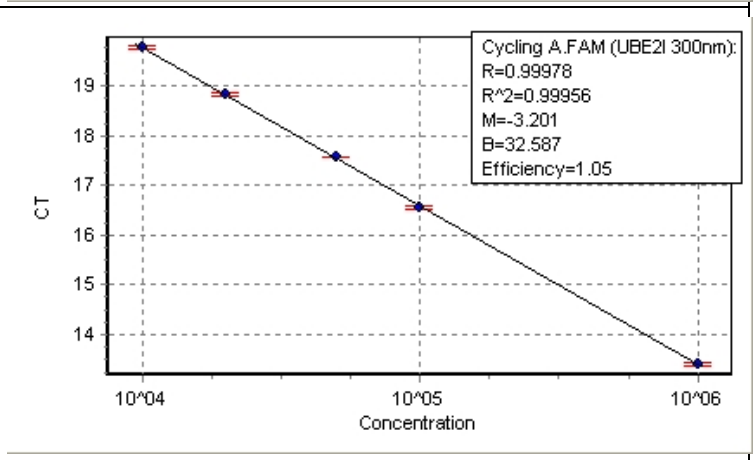
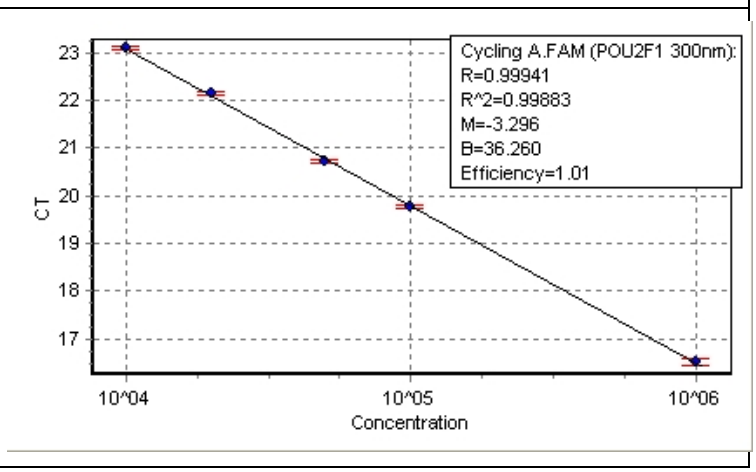
Gene	Primer concentration	Efficiency R^2	Standard curve
Dlx2	200nM	R^2 =0.99 E=1.01	
Pax 7	200nM	R^2 =0.99 E=1.00	

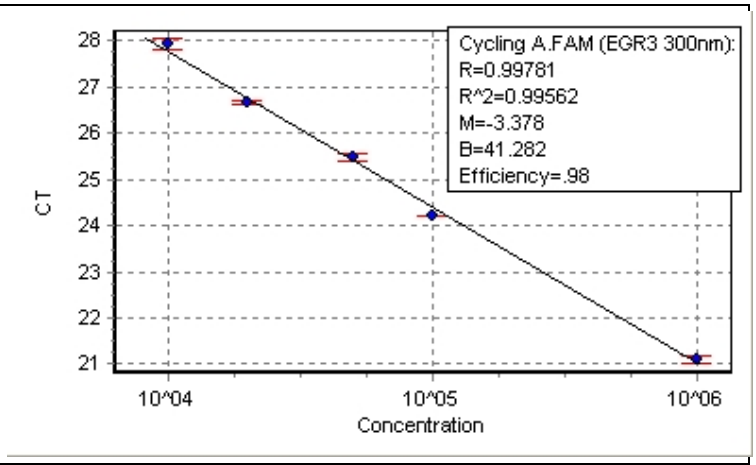
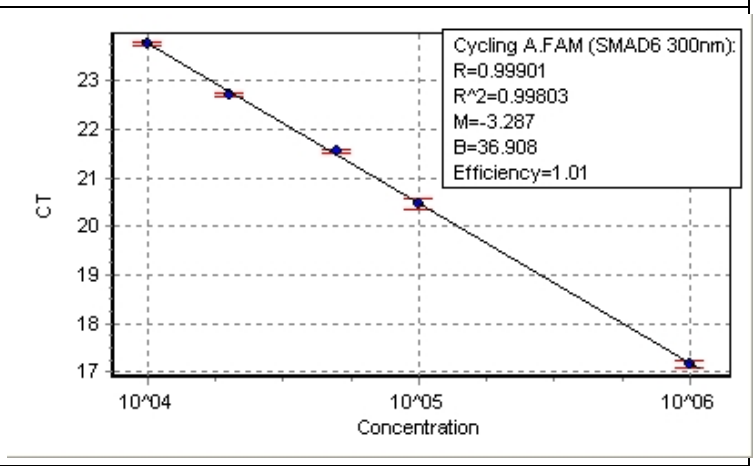
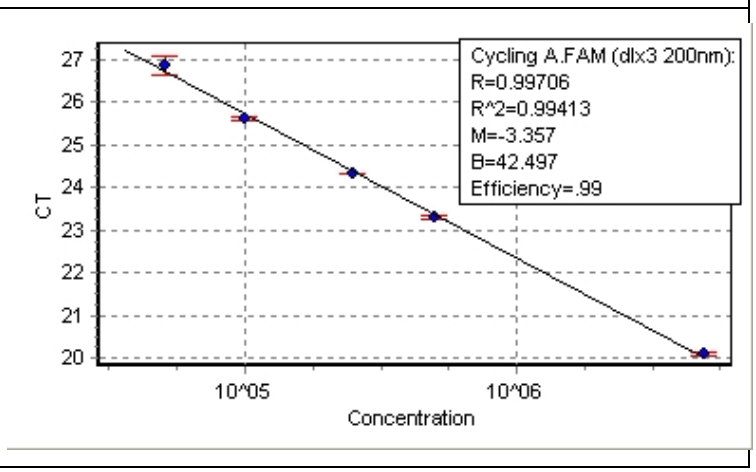
Dlx5	200nM	R^2 =0.99 E=1.05	
Pax 6	200nM	R^2 =0.99 E=1.03	
Pax 3	200nM	R^2 =0.99 E=0.97	

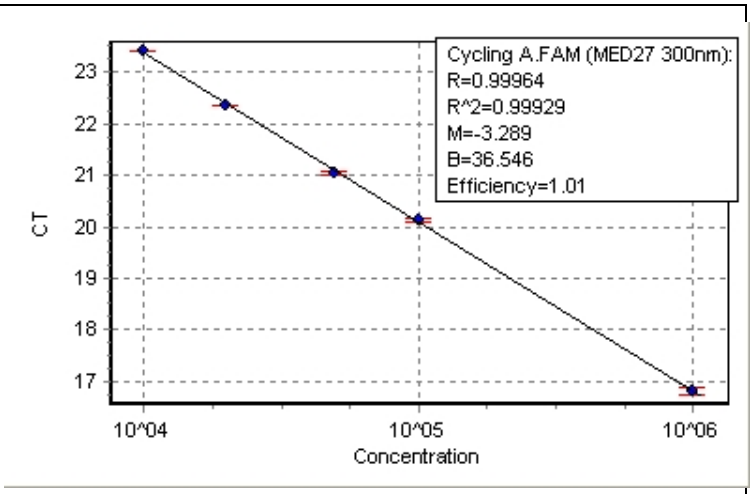
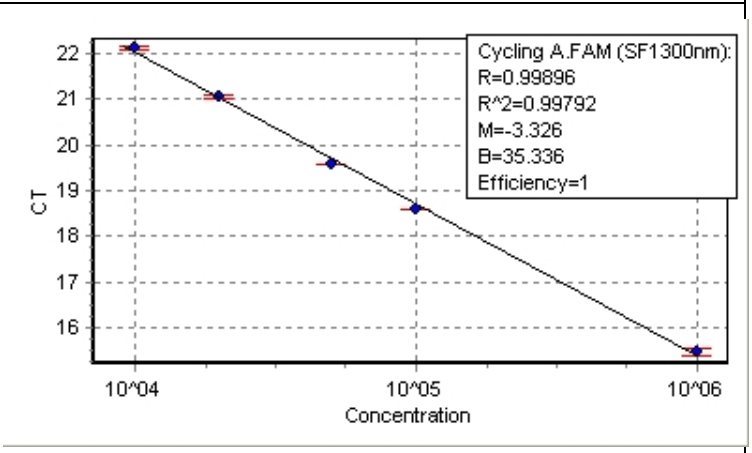
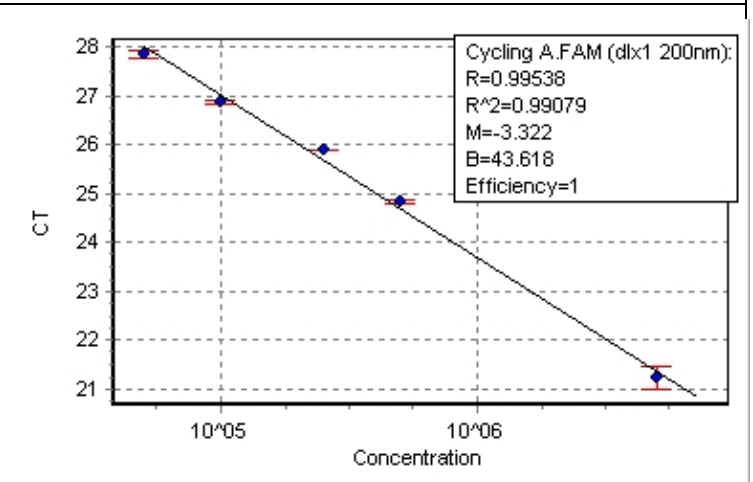
Pou 3f2	200nM	R^2 =0.99 E=0.97	
Hdac9	300nM	R^2 =0.99 E=0.97	
Cxxc4	200nM	R^2 =1.00 E=0.98	

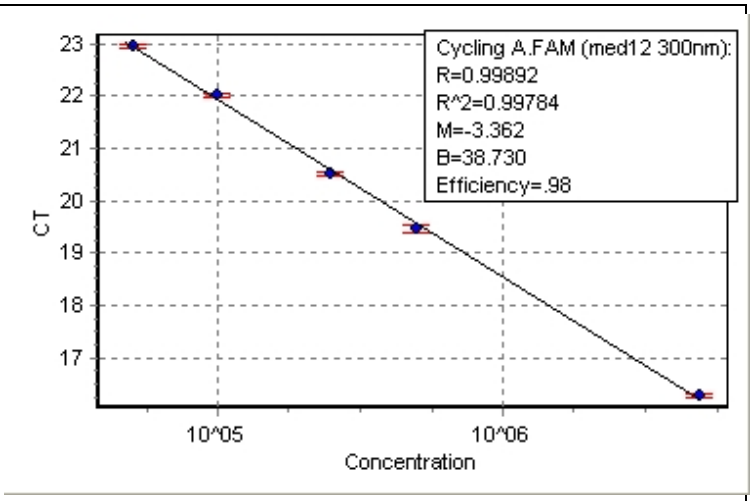
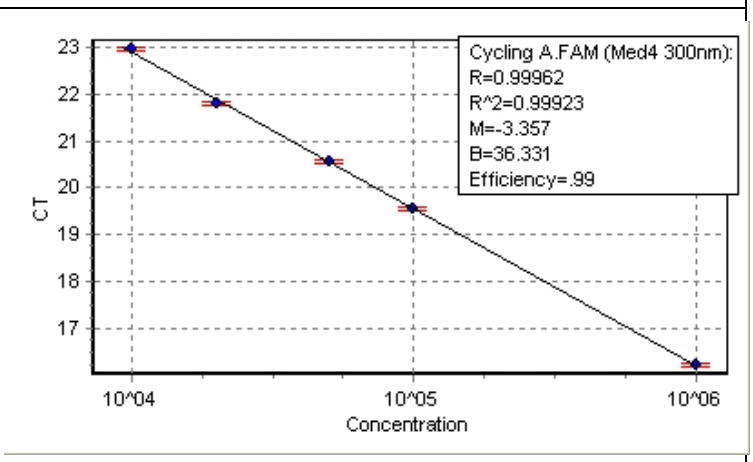
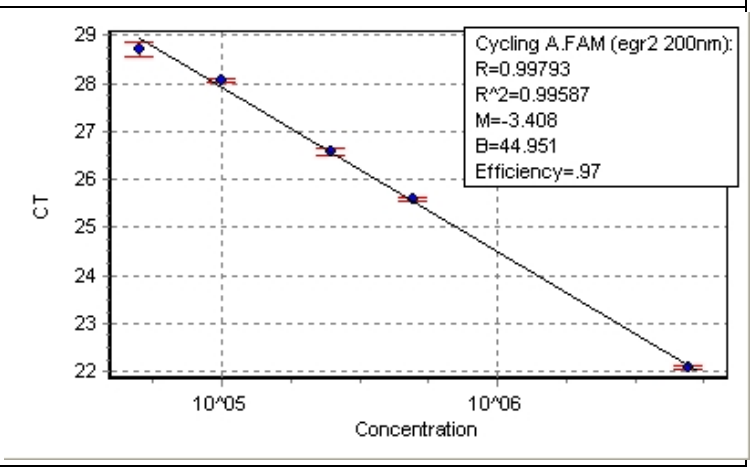
Pou 3f1	200nM	R^2 =0.99 E=0.96	
Hhex	300nM	R^2 =1.00 E=0.95	
Ube 2c	300nM	R^2 =1.00 E=1.00	
Pou 4f1	300nM	R^2 =1.00 E=0.98	

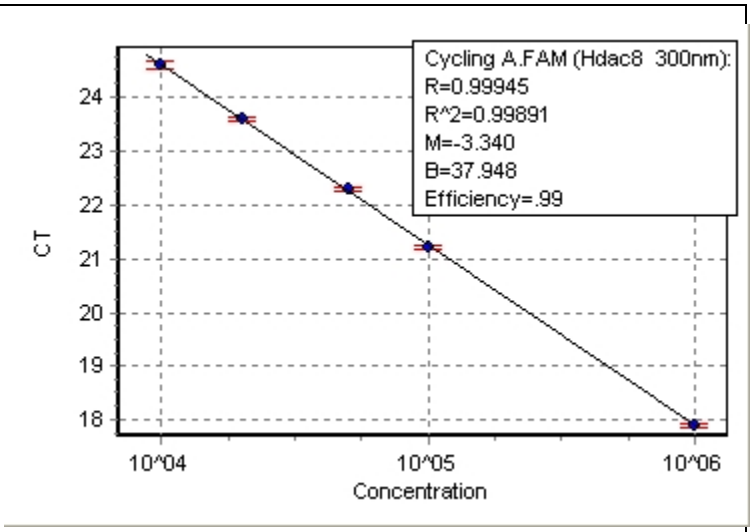
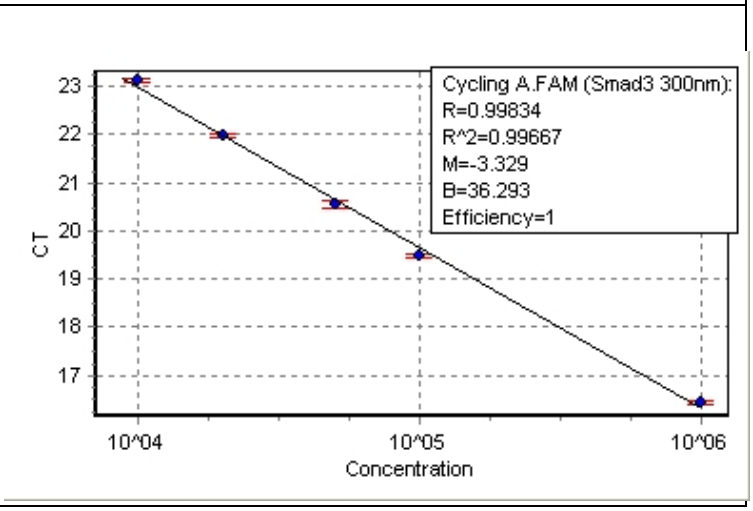
Med 18	300nM	R^2 =1.00 E=1.00	
Egr1	300nM	R^2 =0.99 E=1.00	
Myst 1	300nM	R^2 =1.00 E=1.00	

Hox b4	300nM	R^2 =1.00 E=1.01	
Ube 2i	300nM	R^2 =1.00 E=1.05	
Pou 2f1	300nM	R^2 =1.00 E=1.01	

Egr3	300nM	R^2 =1.00 E=0.98	
Smad6	300nM	R^2 =1.00 E=1.01	
Dlx3	200nM	R^2 =0.99 E=0.99	

Med 27	300nM	R^2 =1.00 E=1.01	
Sf1	300nM	R^2 =1.00 E=1.00	
Dlx1	200nM	R^2 =1.00 E=1.00	

Med 12	300nM	R^2 =1.00 E=0.98	
Med 4	300nM	R^2 =1.00 E=0.99	
Egr2	200nM	R^2 =1.00 E=0.97	

Hdac8	300nM	R^2 =1.00 E=0.99	
Smad3	300nM	R^2 =1.00 E=1	

For the quantitative comparison, each gene analysis was performed on three biological replicates of E12.5, E14.5, E18.5, P7 and P21 murine kidney cDNA in triplicate in a single run using optimized conditions. In addition, *Gapdh*, *Pgk1* and *Hprt1* were evaluated as reference genes using all three biological replicates for each time point. *Pgk1* was determined to be the most stable reference gene using both the NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) algorithms. Therefore, the *Pgk1* was used as the reference gene. Real-time PCR experiments and analyses were conducted following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2010). The threshold of 0.2 was chosen as the default threshold because it was found to be optimal to distinguish signal and background fluorescence.

2.5 Analysis

Relative quantifications were used to compare the expression level of each gene at five time points. The Ct number is the number of PCR amplification cycles required to reach fluorescent intensity above threshold. It was measured for each gene and each developmental time point was analyzed.

The gene expression of different time points was normalized against Pgk1 data by using $\Delta\Delta C_t$ method (Pfaffl et al., 2004) to detemine the relative expression of each shortlisted candidate partners over kidney development, where $\Delta\Delta C_t = ((C_t \text{ (target)} - C_t$

(PGK1))-(Ct (calibrator gene) - C_t (PGK1)). The C_t value of each gene on P21 was used as the calibrator because this value is always the lowest.

To evaluate differences in mean relative quantification of each candidate partner during renal development, one-way ANOVA followed by Tukey's test was performed, using *Microsoft® Excel (v1.10, Microsoft Inc)*. The statistical significance was defined as $p < 0.05$. Graphs were generated using *SigmaPlot (v 5.0, SPSS Science Inc)*.

3. RESULTS

3.1 List of potential SOXC protein partners

A list of candidate protein partner genes was compiled based on the literature and was expanded to include members of the same family based on gene sequence and phylogenetic homology (Table 4).

For example, SOX11 was shown to interact with Pou class 3 homeobox 3 (POU3F3) and Pou class 3 homeobox 2 (POU3F2) in the activation of the Fibroblast growth factor 4 enhancer (FGF4) in the hindbrain (Kuhlbrodt et al., 1998a). The Pou family has a highly conserved DNA binding structure, and is defined as a family of transcriptional regulators (Klemm et al., 1994; Sakurai et al., 2013). Therefore, the POU family proteins encoded by *Pou* genes were assumed to have the potential to interact with SOXC in transcription.

The following families are a few more examples of potential protein partners that were identified based on the literature search.

The Runt-related transcription factor (RUNX) family of proteins are also potential SOXC transcription protein partners. The RUNX proteins are poor activators on their own, and they tend to interact with transcriptional coactivators (Durst and Hiebert, 2004). The *RUNX1* gene is thought to be involved in the development of normal hematopoiesis

(hemopoiesis). Mouse embryos with homozygous mutations of *Runx1* die at about 12.5 days of gestation (Wang et al., 1996; Fujita et al., 2001; Okuda et al., 2001). RUNX2 protein is a key transcription factor associated with osteoblast differentiation (Schroeder et al., 2005) and SOX9 can interact with RUNX2 to repress RUNX2 activity (Zhou et al., 2006). *Runx3* null mice exhibit hyperplasia of the gastric mucosa due to stimulated proliferation and suppressed apoptosis in epithelial cells (Li et al., 2002) and it has also been reported to cooperate with RUNX2 to regulate the differentiation and maturation of chondrocyte (Kim et al., 2013).

Early growth response (EGR) is an immediate-early growth response protein encoded by the *Egr* gene. It participates in the transcriptional regulation of genes and is induced by mitogenic stimulation. SOX10 protein has been shown to interact with EGR2 to regulate *Connexin32* expression in glial cells (Bondurand et al., 2001).

Steroidogenic factor 1 (SF1) is a key component of the pathway of gonadal and adrenal development. The SOX9 protein can bind to an SF1 protein partner to regulate *Amh* gene expression in Sertoli cells (de Santa Barbara et al., 1998; Barrionuevo et al., 2012).

Paired box (PAX) is a family of tissue specific transcription factors. It contains a paired domain and a partial or complete homeodomain. PAX proteins are noteworthy because they are essential for the formation and specification of certain tissues in early animal development. SOX10 protein functions during melanocyte specification and in the melanocyte lineage, but must interact with PAX3 to regulate

microphthamia-associated transcription factor and *c-ret* gene in neural crest cells in order to function successfully (Kuhlbrodt et al., 1998b; Leon et al., 2009).

Distal-less homeobox (DLX) is a family of homeobox transcription factors similar to the *Drosophila distal less* gene. DLX5 has been reported to interact with the HMG domain of SOX10, but the function of the interaction has not been studied (Wissmuller et al., 2006). In the fly, *Dlx2* can control development of the branchial arches and the forebrain (Qiu et al., 1995). However, whether members of the DLX family interact with protein partners, or have a role in murine development is not known.

Table 4. Candidate SoxC signaling partners based on literature search.

Interacting Protein	Sox Family Member	Homologous Genes from Same Family
CtBP (C-terminal binding protein) 2	Sox6 (Murakami et al., 2001)	*Ctbp1*
DLX (Distal-less homeobox) 5	SOX10 (Wissmuller et al., 2006)	*Dlx1,Dlx2, Dlx3, Dlx4, Dlx6*
EGR (Early growth response) 2	SOX10 (Bondurand et al., 2001)	*Egr1, Egr2, Egr3*
EP (E1A binding protein) 300	SOX9 (Cheng et al., 2009)	
HDAC (histone deacetylase) 1	SOX6 (Iguchi et al.,2007)	*Hdac1, Hdac2, Hdac4, Hdac7, Hdac8, Hdac9, Hdac11*
HHEX (hematopoietically expressed homeobox)	SOX13 (Marfil et al., 2010)	
HnRPK (heterogeneous nuclear ribonucleoprotein K)	SOX10 (Melnikova et al., 2000)	
HOX (homeobox) B1	SOX2 (Wilson et al., 2002)	*Hoxa2, Hoxa4, Hoxa6, Hoxa10, Hoxb1, Hoxb4*
MED (mediator complex subunit) 12	SOX9 (Akiyama et al., 2011)	*Med1, Med4, Med6, Med12, Med15, Med17, Med18, Med21, Med23, Med25, Med25, Med27, Med28, Med29, Med30*
MEF (myocyte enhancer factor) 2C	SOX18 (Hosking et al., 2001)	*Mef2a, Med2b, Mef2c, Mef2ds*
NHERF2	SRY (Thevenet et al.,	

	2005)	
OLIG (olig**odendrocyte lineage transcription factor) 2**	SOX10 (Liu et al., 2007)	*Olig1, Olig2, Olig3*
OTX (orthodenticle homeobox) 2	SOX9 (Masuda et al., 2010)	*Otx1, Otx2*
P (tumor protein) 53	SOX4 (Hur et al., 2010)	*P53, P63, P73*
PAX (paired box) 3	SOX10 (Kuhlbrodt et al., 1998b)	*Pax2, Pax3, Pax4, Pax5, Pax6, Pax7, Pax8*
PGC(Peroxisome Proliferator-Activated Receptor Gamma, Coactivator) 1a	SOX6 (Liao et al., 2010)	*Pgc1b*
POU (pou class) 3f3	SOX11 (Kuhlbrodt et al., 1998a)	*Pou1f1, Pou2f1, Pou2f3, Pou3f1, Pou3f2, Pou3f3, Pou4f1, Pou4f2, Pou4f3, Pou5f1*
RUNX (runt-related transcription factor) 2	SOX9 (Zhou et al. 2006)	*Runx1, Runx2, Runx3*
SF (splicing factor) 1	SOX9 (de Santa Barbara et al., 1998)	
SMAD1	SOX9 (Kim and Lassar, 2003)	*Smad1, Smad2, Smad3, Smad4, Smad5, Smad6, Smad7, Smad9*
TIP60 (K(lysine) acetyltransferase)	SOX9 (Hattori et al., 2008)	*Myst2, Myst3, Cbp*
UBE (ubiquitin-conjugating enzyme) 2I	SOX4 (Pan et al., 2006)	*Ube2a, Ube2b, Ube2c, Ube2d1, Ube2d2, Ube2d3, Ube2f, Ube2g1, Ube2h, Ube2i, Ube2j1, Ube2j2, Ube2k*

3.2 RT-PCR

This first step resulted in a short list of **28** SoxC signaling candidate partners which are highly developmentally regulated in the murine kidney. These include *Pax7, Dlx5, Pax4, Pax6, Pou4f3, Pou4f2, Dlx4, Pou3f2, Hdac9, Cxxc4, Pou3f1, Hhex, Ube2c, Pou4f1, Med18, Egr3, Smad6, Dlx3, Med27, Sf1, Dlx1, Med12, Med4, Egr2, Hdac8* and *Smad3* (Table 5).

Table 5. Fold change of expression level from E13.5 to P21 of short-listed candidate SOXC signaling partners.

Candidate SOXC signaling partner	Fold change (E13.5/P21)
Pax7	493
Dlx5	452.5
Pax4	293.25
Pax6	247.25
Pou4f3	242.75
Pax3	224
Pou4f2	113
Dlx4	85.25
Pou3f2	50.5
Hdac9	14.15116
Cxxc4	4.671576
Pou3f1	4.231915
Hhex	3.242424
Ube2c	3.042424
Pou4f1	2.938499
Med18	2.678788
Egr1	2.674051
Myst1	2.556465
Hoxb4	2.429379
Ube2i‚26	1.991266
Pou2f1	1.920755
Egr3	1.850987
Smad6	1.824271
Dlx3	1.757213
Med27	1.681951
Sf1	1.551515
Dlx1	1.521682
Med12	1.495323
Med4	1.492754
Egr2	1.462827
Hdac8	1.428571
Smad3	1.401124

3.3 Realtime PCR

Real time qPCR was used to analyze the mRNA expression patterns of short listed candidate protein partners in order to identify those that are developmental expressed during renal development and to identify protein partners that have a similar mRNA expression pattern as the *SoxC* subfamily members.

Based on a primer efficiency test, *Dlx4, Dlx6, Pax4, Pou4f2* and *Pou4f3* were excluded out of the list of potential SOXC signaling partners because the expression levels were too low to have eligible primer efficiencies. Real-time PCR analysis using cDNA from murine kidneys at E12.5, E14.5, E18.5, P7 and P21 was performed to study the expression pattern of the SOXC candidate partners. From the 28 candidate partners analyzed, several genes showed similar expression patterns to the *SoxC* subfamily.

Based on the real-time PCR showing the mRNA expression pattern of each short listed potential protein partner of SOXC subfamily, *Dlx2, Dlx3* and *Dlx5* from the *Dlx* family (Fig. 8), *Pou3f1*and *Pou3f2* from the *Pou* family (Fig. 9), *Pax3*, *Pax6* and *Pax7* from the *Pax* family (Fig. 10), *Ube2i* and *Ube2c* from the *Ube* family (Fig. 11), *Med4*, *Med12*, *Med18* and *Med21* from the *Med* family (Fig. 12), *Cxxc4* (Fig. 13) and *Sf1* (Fig. 14) showed similar mRNA expression patterns as *SoxC* subfamily members. In contrast, the mRNA expression pattern of *Egr1*, *Egr2*, *Egr3*, *Smad3*, *Smad6*, *Hdac8*, *Hdac9*, *Hoxb4* and *Hhex* was dissimilar to that of the *SoxC* subfamily (Fig. 15).

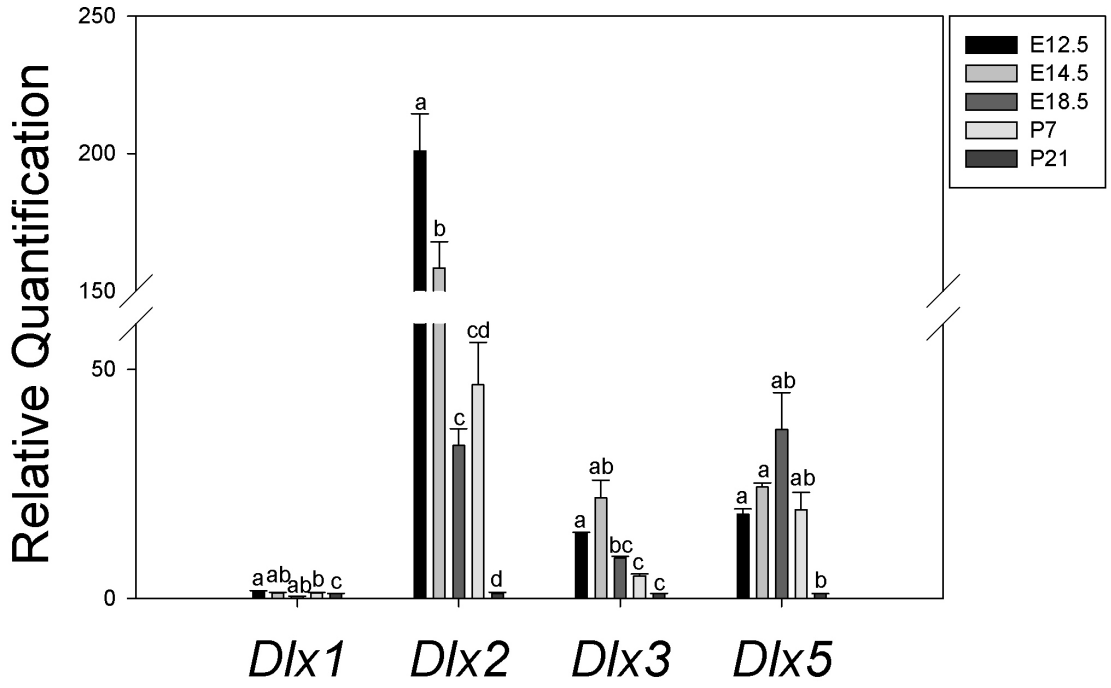


Figure 8. Developmental expression of *Dlx1*, *2*, *3* and *5* in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

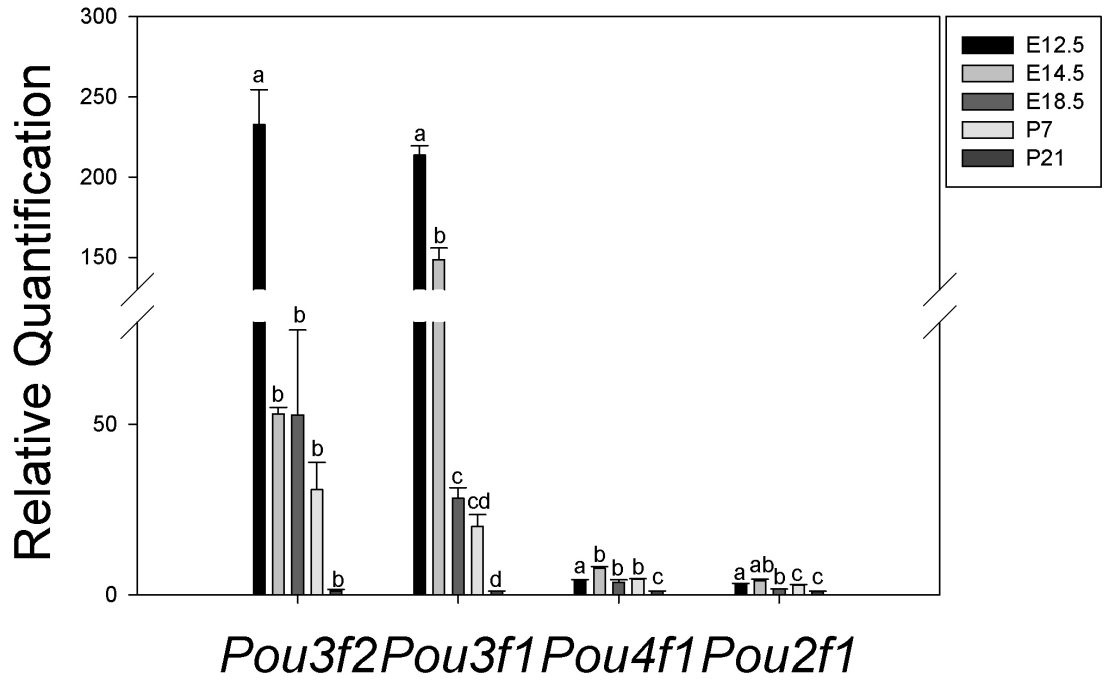


Figure 9. Developmental expression of *Pou class 3 homeobox 2 (Pou3f2)* and *1 (Pou3f1), Pou class 4 homeobox1(Pou4f1)* and *Pou class 2 homeobox1(Pou2f1)* in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

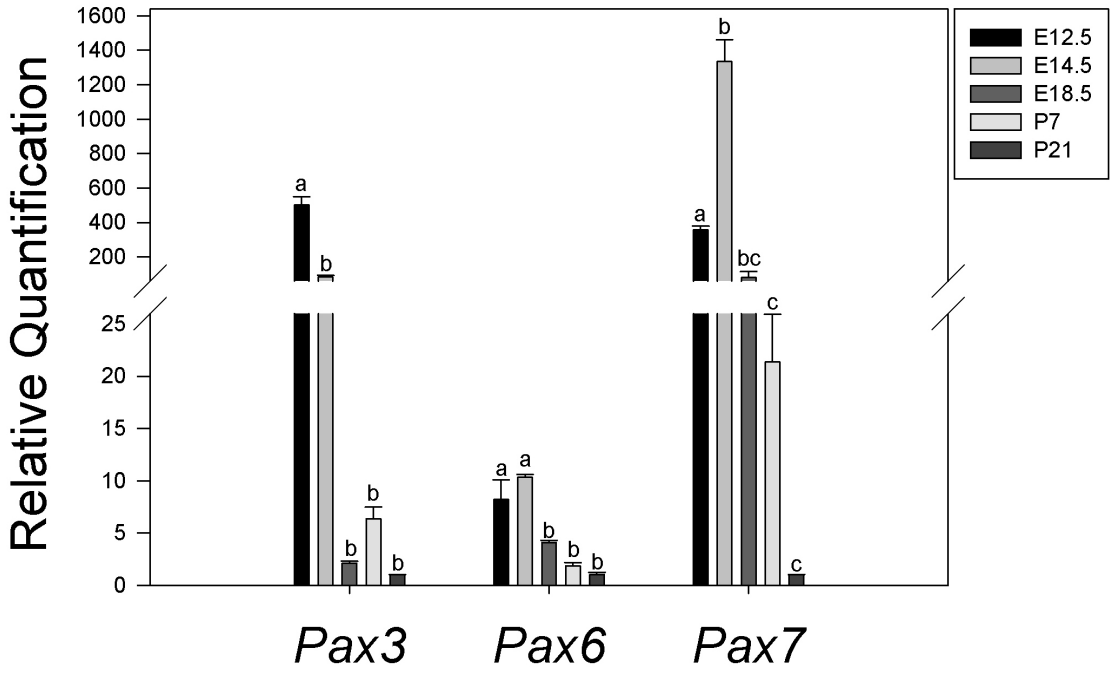


Figure 10. Developmental expression of *paired box (Pax) 3, 6* and *7* in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

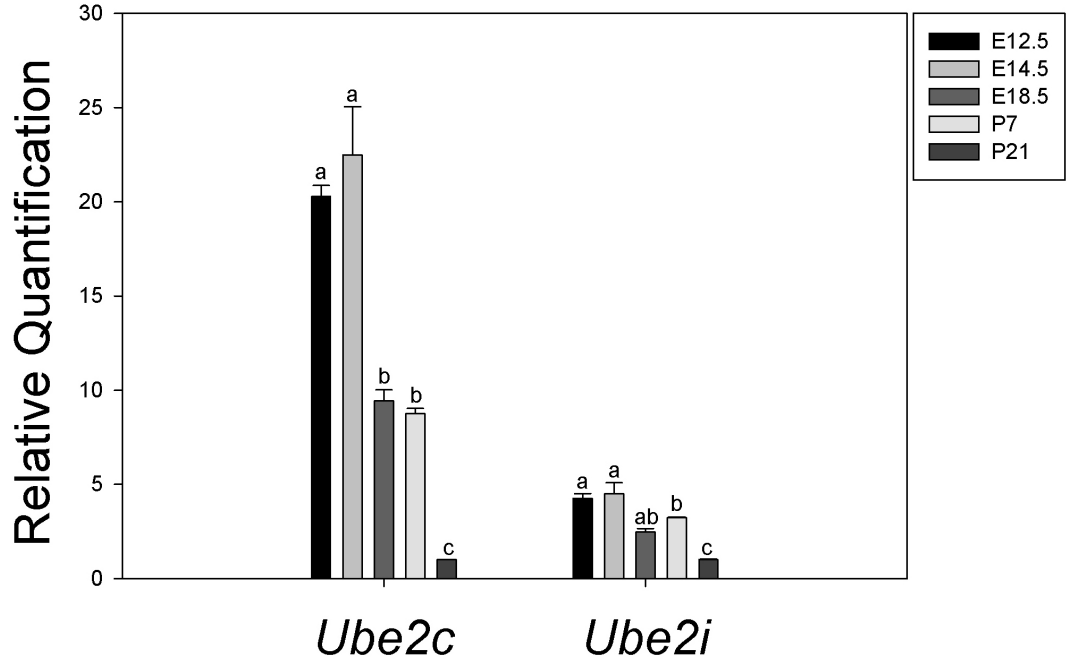


Figure 11. Developmental expression of *Ubiquitin-conjugating enzyme E2C (Ube2c)* and *E2I (Ube2i)* in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

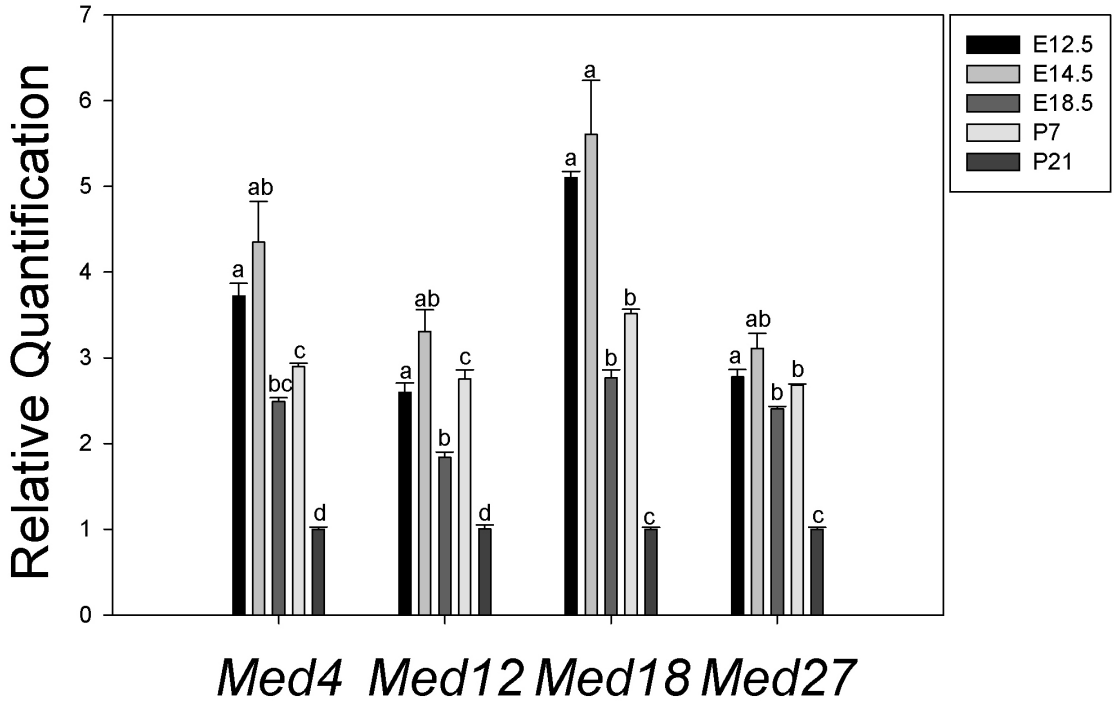


Figure 12. Developmental expression of ***med****iator complex subunit (Med) 4, 12, 18* and *27* in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

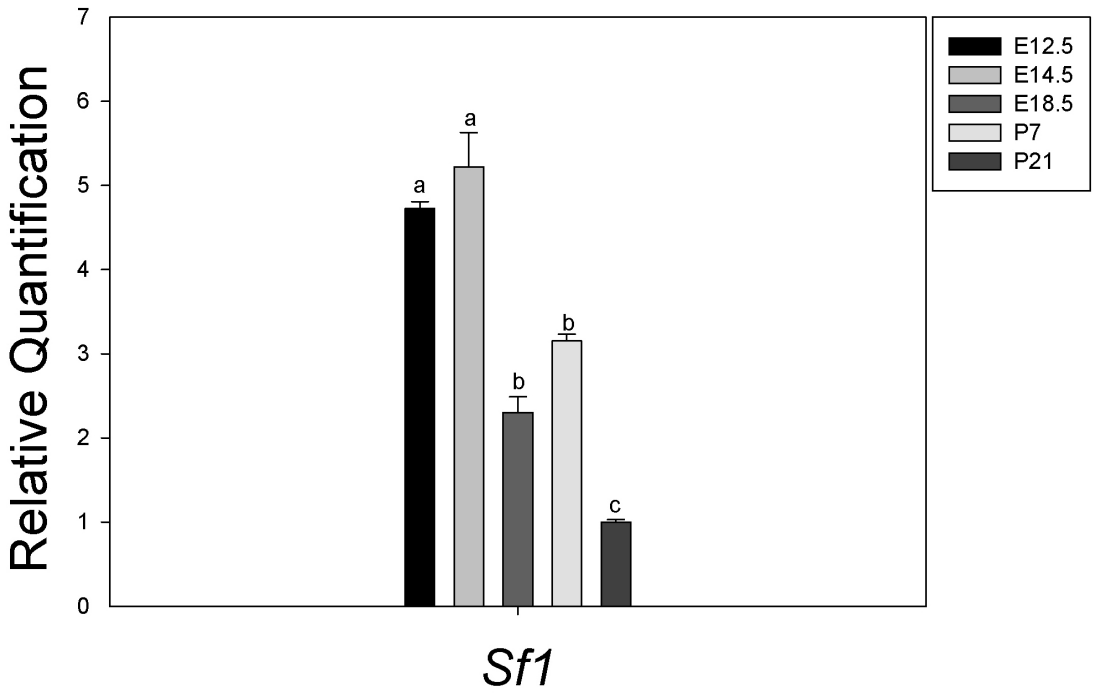


Figure 13. Developmental expression of *splicing factor 1(Sf1)* in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

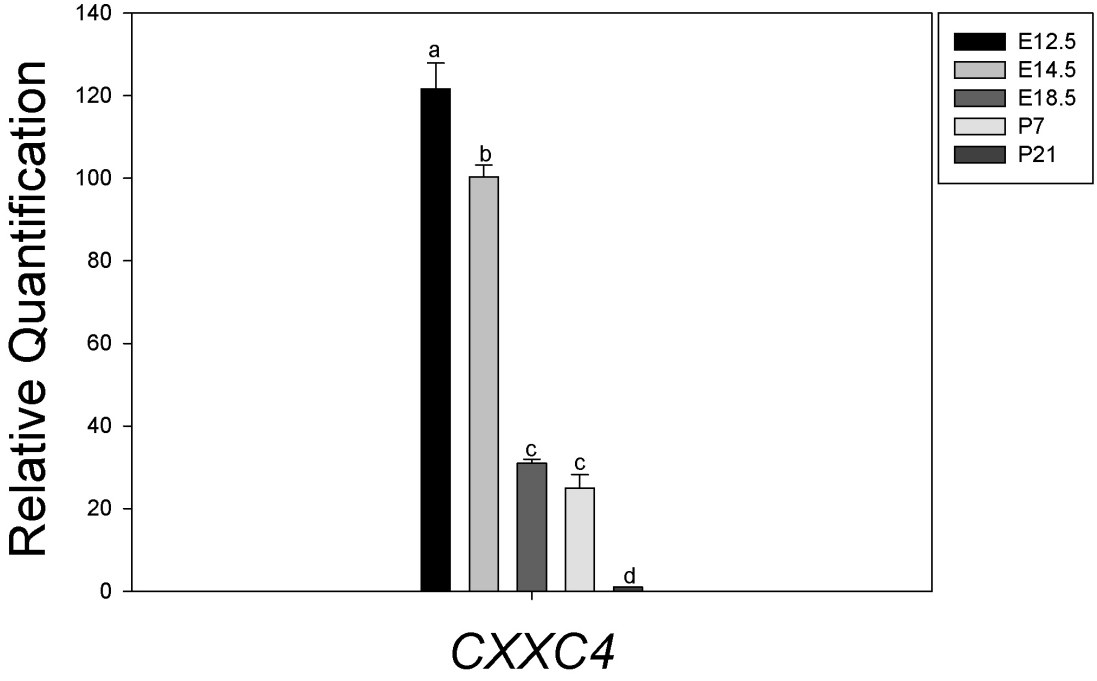


Figure 14. Developmental expression of *CXXC finger protein 4(Cxxc4)* in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

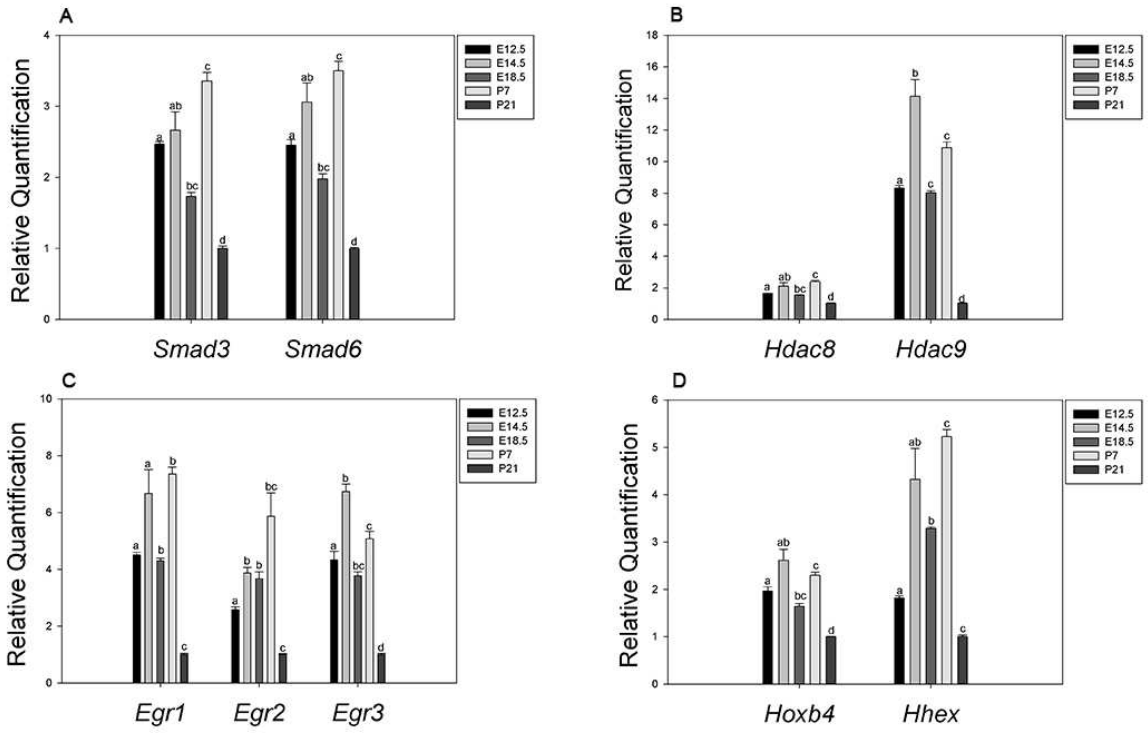


Figure 15. (A-D) Expression of *Smad3,6* (A), *Hdac8,9* (B), *Egr1,2,3*(C) *Hoxb4* and *Hhex* (D) in E12.5, E14.5 E18.5, P7 and P21 murine kidney measured by real-time PCR. Each data point is calibrated against the expression of each gene at P21 using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard error (n=3). Differences among the letters above the bars indicate a significant ($P < 0.05$) difference from one-way ANOVA followed by Tukey's test.

4. DISCUSSION

4.1 The shortlist of SOXC candidate partners

mRNAs of all three members of the *SoxC* subfamily have been found to be developmentally regulated in the kidney, with greater expression of *SoxC* mRNA in the early stages of renal development and decreasing expression in later stages (Huang et al., 2013). SOX protein partners are believed to increase stability of SOX transcription factors binding on target genes whether physically bound to Sox transcription factors or not (Kondoh and Kamachi, 2010), and it is believed that potential protein partners of SOXC transcription factors have similar expression patterns as the transcription factors with which they partner. Based on the pixel intensity of all tested candidate SOXC partners gel electrophoresis images, 28 candidate partners were short listed for quantitative analysis. *Collagen type II, alpha 1 (Col2a1)* was excluded from the short list, because *Col2a1* encodes the pro-alpha (II) chain, which is one component of type II collagen. Type II collagen provides the structure and strength to connective tissues and is mainly found in cartilage (Chan et al., 1995) and *Col2a1* is expressed in the renal hilum and renal pelvis. It is believed that COL2A1 functions in connective tissue formation in the kidney but not nephrogenesis.

4.2 *Dlx* family expression

The DLX family of proteins participate in a variety of developmental processes in the mouse such as neurogenesis and hematopoiesis (Panganiban et al., 2002). The mRNA expression pattern of *Dlx2* (Fig. 8) was similar to *Sox4* mRNA expression (Huang et al., 2013), in that the expression was greatest in the earlier stages of the renal development (E12.5) and then decreased with advancing renal development, with the exception of a peak at P7 (Fig. 8). *Dlx2* and *Dlx5* come from the same clade of the phylogenetic tree (Stock et al., 1996), and they play similar roles in mouse lens development (Kwakowsky et al., 2007), which suggests that they have high developmental relevance during lens development. DLX5 has been reported to interact at the protein level with SOX10 (Wissmuller et al., 2006). Because of the similarity to DLX5, DLX2 may also interact with SOX proteins. For these reasons, both DLX2 and DLX5 are considered to have a high potential of being protein partners of SOXC transcription factors.

Dlx3 mRNA expression pattern was similar to both *Sox11* and *Sox12* (Huang et al., 2013), with greater expression in the early stages of kidney development (Fig. 8). *Dlx3* is expressed in the nephrogenic zone where *SoxC* mRNA is expressed at E14.5 in the murine kidney (Genepaint, 2003), therefore DLX3 has a high potential of being a protein partner of the SOXC transcription factors. Since *Dlx1* did not show a similar mRNA expression pattern to the *SoxC* subfamily (Fig. 8), it was excluded as a potential

SoxC protein partner.

4.3 *Pou* family expression

The Pou domain protein family was derived from three transcription factors; PIT-1, OCT-1as well as the nematode protein UNC-86 (Latchman, 1999; Lodato et al., 2013). The POU family members have various functions, such as those related to the neuroendocrine system (Lodato et al., 2013). Based on the real-time PCR data the *Pou3f1* and *Pou3f2* shared similar expression patterns with *Sox11* and *Sox12* (Huang et al., 2013), where there is a significant decreasing expression throughout renal development (Fig. 9). The Pou domain of POU3F1 (also called Octamer-binding protein 7) was reported to be capable of replacing the Pou domain of OCT3/4. The Pou domain of OCT 3/4 is essential for its ability to bind to the SOX2 transcription factor to regulate UTF (Undifferentiated embryonic cell transcription factor) 1 in pluripotent embryonic cells through the Pou domain (Nishimoto et al., 1999). Since OCT3/4 can interact with the Sox2 transcription factor, there is a possibility that POU3F1 is a protein partner of the SOXC transcription factors. In addition, according to *in situ* hybridization data, there is significant expression of *Pou3f1* in the nephrogenic zone in murine kidney at E14.5 (Genepaint, 2003) where *SoxC* mRNA is expressed. Therefore, POU3F1 has the potential to be a SOXC signaling partner.

For POU3F2 (Brain-specific homeobox/POU domain protein 2), there is

evidence showing that all three members of the SOXB1 subfamily (SOX1, SOX2 and SOX3) can play a role as DNA binding proteins to interact with POU3F2 to regulate the *Nestin* geneural primordial cells (Tanaka et al., 2004). Also, the expression of *Pou3f2* is reported in NPC in murine kidney at E15.5 (Gray et al., 2004). Because *Sox4* and *Sox12* mRNA have been shown to be expressed in NPC and ureteric bud tips during murine renal development (Huang et al., 2013), POU3F2 is considered a potential SoxC signaling partner. Since *Pou4f1* and *Pou2f1* did not show similar mRNA expression patterns to the *SoxC* subfamily in the developing kidney (Fig. 9), they were excluded as potential SOXC signaling partners.

4.4 *Pax* family expression

Pax3 has been reported as being expressed in the stromal cells and nephron progenitor cells of the developing kidney (Hueber et al., 2009). It has been shown that SOX10 and PAX3 can physically interact to mediate the conserved c-RET enhancer. This also supports the notion that PAX3 can be a SOXC signaling partner because *c-RET*, which is expressed in the ureteric bud tips, can cooperate with WNT11 and GDNF to regulate ureteric branching during renal development (Majumdar et al., 2003; Leon et al., 2012) and *Sox4*/*Sox12* are expressed at ureteric bud tips (Huang et al., 2013). Therefore it is possible that the SOXC subfamily interacts with PAX3 in the regulation of *c-RET*. Since the mRNA expression pattern of *Pax3* (Fig. 10) is similar to the

expression pattern of Sox4 in renal development (Huang et al., 2013), PAX3 is a possible signaling partner of SOXC transcription factors.

Pax7 and *Pax6* mRNAs expression also showed similar patterns (Fig. 10) to *Sox11* and *Sox12* in renal development (Huang et al., 2013). PAX6 protein has been reported to directly and positively regulate its own gene expression as well as *Sox2* expression (Aota et al., 2003). Furthermore, SOX3 protein can interact with PAX6 to activate the head surface ectoderm-specific enhancer of *Pax6* synergistically with PAX6 (Aota et al., 2003; Modrell et al., 2011). Also, there is evidence showing that PAX6 can interact with SOX10 via its DNA-binding domain in a yeast two-hybrid screen (Wissmuller et al., 2006). Although there is no prior evidence to support *Pax6* and *Pax7* expression in renal development, there is still a possibility that PAX6 and PAX7 are SOXC signaling partners since the online database examined *Pax6* and *Pax7* expression at one or two time points during renal development. The spatiotemporal expression of *Pax6* and *Pax7* during mouse kidney development will be identified by *in situ* hybridization in future work.

4.5 *Ube* family expression

UBE2I (ubiquitin conjugating enzyme 9) has been identified as a SOX4-interacting protein by yeast two-hybrid screen (Pan et al., 2006). *Ube2i* was also

shown to be co-localized with *Sox4* in the nucleus of living cells by fluorescence microscopy analyses. In addition, the HMG domain, which usually mediates the interactions between SOX transcription factors and partner proteins, was found to be required for the interaction between SOX4 and UBE2I. UBE2I has also been identified as participating in the repression of SOX4 transcriptional activity in a human breast cancer cell line (Pan et al., 2006). Although the mechanisms by which UBE2I represses the transcriptional activity of SOX4 are still unknown, UBE2I is believed to act as a protein partner of SOX4. The expression pattern of *Ube2i* mRNA (Fig. 11) during murine renal development is similar to the expression pattern of *Sox4* mRNA (Huang et al., 2013). *Ube2i* mRNA expression has also been detected in the nephrogenic zone of the murine kidney at E14.5 by *in situ* hybridization (Genepaint, 2003). This evidence supports the notion that UBE2I may play a repression role as it partners with Sox4 in mouse kidney development.

There is no current evidence supporting the suggestion that the UBE2C protein interacts with other transcription factors. However, the expression pattern of *Ube2c* mRNA (Fig. 11) was similar to the expression pattern of *Sox11* and *Sox12* mRNA (Huang et al, 2013), and there is significant *Ube2c* expression at the nephrogenic zone where the *SoxC* subfamily is expressed based on *in situ* hybridization (Genepaint, 2003). Though there is no direct evidence of an Ube2c/SoxC interaction reported, I cannot exclude the possibility that Ube2c is a protein partner of SoxC signalling.

4.6 *Med* family expression

The Mediator complex (MED) family interacts with the RNA polymerase II enzyme and is considered a transcription cofactor and a global regulator of transcription with gene-selective functions (Taatjes, 2010). For example, MED1 is able to activate the transcription of thyroid hormone receptor (TR)-regulated promoters (Pandey et al., 2005). MED12 (also called thyroid hormone receptor-associated protein, 230 kDa subunit) has been reported to interact with SOX9 both *in vitro* and *in vivo* and, by immunofluorescence, to co-localize with *Sox9* in human embryonic chondrocytes (Zhou et al., 2006). The C-terminal domain of MED12 can also inhibit SOX9-mediated activation, suggesting that an interaction with MED12 is required for the biological function of SOX9 (Zhou et al., 2002). MED 12 has also been reported to interact with G9A methyltransferase to regulate a subset of neuronal genes (Taatjes, 2010). *Med12* expression is detected in the nephrogenic zone of the murine kidney at E14.5 where *SoxC* expression is detected by *in situ* hybridization (Genepaint, 2003). With the mRNA expression pattern of *Med12* (Fig. 12) being similar to the mRNA expression pattern of *Sox4*, it can be concluded that MED12 is a significant candidate SOXC signaling partner.

Med27 also shares a similar mRNA expression pattern (Fig. 12) as *Sox4* mRNA expression pattern (Huang et al., 2013). *Med27* expression was detected in the ureteric bud tips of murine kidney at E14.5/E15.5 co-localized with *SoxC* expression

from the *in situ* hybridization data (Genepaint, 2003; Gray et al., 2004). However, there is no evidence reported that MED27 can interact with SOX transcription factors. Since *Med27* shares a similar mRNA expression pattern as *Sox4* expression in both real-time PCR and *in situ* hybridization, MED27 will still be classified as a candidate partner of SOXC transcription factors.

Med4 and *Med18* also share similar mRNA expression patterns (Fig. 12) as *Sox4* mRNA (Huang et al., 2013). *Med4* and *Med18* expression is detected in the nephrogenic zone of murine kidneys at E14.5 where *SoxC* is expressed (Genepaint, 2003). However, there is no evidence reported that MED4 and MED18 can interact with SOX transcription factors. Since *Med4* and *Med18* share a similar mRNA expression pattern as *Sox4* in both real-time PCR and *in situ* hybridization, MED4 and MED18 will be chosen as candidate partners of SOXC transcription factors.

4.7 Expression of other genes

The mRNA expression pattern of *Sf1* (Fig. 13) is similar to that of *Sox4* (Huang et al., 2013), where the expression was greatest in the earlier stages of the mouse renal development (E12.5) and then decreased throughout development with the exception of a peak at P7. SF1 was also reported to cooperate with SRY to up-regulate *Sox9* e expression, and SOX9 also binds to the enhancer of *Sf1* to regulate its own expression

during murine gonad formation (de Santa Barbara et al., 1998; Barrionuevo et al., 2012). *Sf1* mRNA expression was also detected in the ureteric bud tips at E15.5 mouse kidney where *Sox4* and *Sox12* were expressed based on the GUD map (Gray et al., 2004) and Genepaint (2003). This supports the notion of SF1 being a potential protein partner of SOXC transcription factors.

CXXC4, also called IDAX, has been reported as a DVL binding protein to repress the WNT dependent beta-catenin accumulation in mammals (Hino et al., 2001). WNT/beta-catenin signaling is critical for nephrogenesis. There is evidence showing that two WNT members, WNT4 and WNT9B, can induce the formation of renal vesicles via the canonical WNT signaling pathway and that this requires beta-catenin (Park et al., 2007). The SOX4 transcription factor has been reported to bind to and stabilize beta-catenin in canonical WNT signaling (Kormish et al., 2010). From the real-time PCR data, the mRNA expression pattern of *Cxxc4* (Fig. 14) is similar to the expression pattern of *Sox11* and *Sox12* (Huang et al., 2013). We conclude that there is a possibility that CXXC4 is a protein partner of SOXC transcription factors.

4.8 Genes that did not show similar expression pattern to SOXC subfamily

Expression analysis of *Egr1, Egr2, Egr3, Smad3, Smad6, Hdac8, Hdac9, Hoxb4* and *Hhex* in embryonic and postnatal renal development (Fig. 15), showed mRNA expression patterns that differed when compared to the *SoxC* subfamily (Huang et al.,

2013). Since the signaling partners of SOXC transcription factors should share similar mRNA expression pattern as the SOXC transcription factor in order to interact, these genes were excluded as candidate SOXC signaling partners.

The limitation of this work is that there is still a possibility that the genes with low expression pattern are partners of SOXC transcription factors. However, since the accuracy of real time PCR was low in those genes expressed low during murine renal development, I have to exclude those genes due to the technical defects. My work is an initial study providing fundamental information necessary for further study of interaction of SOXC protein partners and SOXC transcription factors.

5. CONCLUSIONS

5.1 Summary

Based on my data and the literature search, DLX2, DLX3, DLX5, POU3F1, POU3F2, PAX3, PAX6, PAX7, UBE2I, UBE2C, MED4, MED12, MED18, MED21, CXXC4 and SF1 all have high potential to be protein partners of SOXC transcription factors during nephrogenesis. Their interactions could play a role in the control of nephrongenesis during murine renal development. In the near future, co-localization and functional interaction between SOXC transcription factors and their protein partners will be explored. This will allow for a comprehensive understanding of the SOXC regulatory network during kidney development.

Overall, my research provides the foundation for an understanding of protein partners of SOXC transcription factors and will allow us to comprehensively understand the SOXC regulatory network during the kidney development *in vivo*. It also provides a new understanding of the molecular mechanisms of the WT1 signal network during nephrogenesis and equips us with a starting point from which to design therapies for *de novo* tissue regeneration based on renal stem cells which could reverse the progression of several kidney diseases.

5.2 Future Directions

The current investigation represents the quantitative expression pattern of potential SOXC transcription factors protein partners. The next step will focus on localization of these protein partners during murine renal development. Indirect immunofluorescence double staining or consecutive mRNA *in situ* hybridization will be performed to co-localize potential partners and transcription factors. Since protein partners are believed to either bind to SOX transcription factors or bind to the same target gene as SOX transcription factors at the close binding sites (Kamachi et al., 2000), protein partners and SOXC transcription factors are believed to be expressed in the same structures during murine renal development. This will allow us to narrow down the list of potential SOXC transcription factors protein partners. Since conditional ablation of SOX4 function in the NPC (*Sox4* NPC-/-) will cause defects of murine glomeruli formation (Huang et al., 2013), analyses of compound *SoxC*- and potential partners-conditional ablation of function in the NPC can be used to identify the functional interaction between SOXC transcription factors and potential partners. Also, yeast two-hybrid screen can be used to show the physical interaction of SOXC transcription factors and their protein partners. Chromatin immunoprecipitation assay or DNA pull down assay can be used to identify SOX/Protein partners/target genes interaction.

6. REFERENCES

Akiyama H. 2011. Transcriptional regulation in chondrogenesis by Sox9. Clin. Calcium 21: 845-851.

Andersen CL, Jensen JL, Orntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64:5245-5250.

Andersson T, Södersten E, Duckworth JK, Cascante A, Fritz N, Sacchetti P, Cervenka I, Bryja V, Hermanson O. 2008. CXXC5 is a novel BMP4-regulated modulator of Wnt signalling in neural stem cells. J. Biol. Chem. 284:3672–3681.

Aota S, Nakajima N, Sakamoto R, Watanabe S, Ibaraki N, Okazaki K, 2003. Pax6 autoregulation mediated by direct interaction of Pax6 protein with the head surface ectoderm-specific enhancer of the mouse Pax6 gene. Dev. Biol. 257:1-13.

Barrionuevo FJ, Burgos M, Scherer G, Jimenez R. 2012. Genes promoting and disturbing testis development. Histol Histopathol 27:1361-1383.

Bergsland M, Werme M, Malewicz M, Perlmann T, Muhr J. 2006. The establishment of neuronal properties is controlled by SOX4 and SOX11. Genes Dev. 20:3475–3486.

Bernard P, and Harley VR. 2010. Acquisition of SOX transcription factor specificity through protein-protein interaction, modulation of Wnt and post- translational modification. Int. J. Biochem. Cell Biol. 42:400-410.

Bondurand N, Girard M, Pingault V, Lemort N, Dubourg O, Goossens M. 2001. Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. Hum. Mol. Genet. 10:2783-2795.

Bouchard M, Pfeffer P, Busslinger M. 2000. Functional equivalence of the transcription factors Pax2 and Pax5 in mouse development. Development 127: 3703–3713.

Bowles J, Schepers G, Koopman P. 2000. Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev. Biol. 227:239-255.

Breslow N, Olshan A, Beckwith JB, Green DM. 1993. Epidemiology of Wilms tumor. Med. Pediatr. Oncol. 21:172–181.

Bridgewater D, and Rosenblum ND. 2009. Stimulatory and inhibitory signaling molecules that regulate renal branching morphogenesis. Pediatr. Nephrol. 24:1611-1619.

Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, Penning LC, Toegel S. 2010. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol. Biol. 11:74

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, Housman DE. 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60:509–520.

Castillo SD and Sanchez-Cespedes M. 2012. The SOX family of genes in cancer development: Biological relevance and opportunities for therapy. Expert Opin. Ther. Targets 16:903-919.

Chai OH, Song CH, Park SK, Kim W, Cho ES. 2013. Molecular regulation of kidney development. Anat. Cell Biol. 46:19-31.

Chan D, Cole WG, Chow CW, Mundlos S, Bateman JF. 1995. A COL2A1 mutation in achondrogenesis type II results in the replacement of type II collagen by type I and III collagens in cartilage. J. Biol. Chem. 270:1747-1753.

Chau YY and Hastie ND. 2012. The role of Wt1 in regulating mesenchyme in cancer, development, and tissue homeostasis. Trends Genet. 28:515-524.

Cheng CC, Uchiyama Y, Hiyama A, Gajghate S, Shapiro IM, Risbud MV. 2009. PI3K/AKT regulates aggrecan gene expression by modulating Sox9 expression and activity in nucleus pulposus cells of the intervertebral disc. J. Cell Physiol. 221:668-676.

Collignon J, Sockanathan S, Hacker A, Cohen-Tannoudji M, Norris D, Rastan S. 1996. A comparison of the properties of SOX-3 with Sry and two related genes, SOX-1 and SOX-2. Development 122:509–520.

Costantini F. 2010. Renal branching morphogenesis: Concepts, questions, and recent advances. Differentiation 74:402-421.

Davidson AJ. Mouse kidney development. 2009 Jan 15. In: StemBook [Internet]. Cambridge (MA): Harvard Stem Cell Institute; 2008-.Available from: http://www.ncbi.nlm.nih.gov/books/NBK27080/

De Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Südbeck P, Scherer G, Poulat F, Berta P. 1998. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Müllerian hormone gene. Mol. Cell Biol. 18:6653-6665.

Denys P, Malvaux P, van den Berghe H, Tanghe W, Proesmans W. 1967. Association d'un syndrome anatomo-pathologique de pseu- do-hermaphrodisme masculin, d'une tumeur de Wilms, d'une nephropathie parenchymateuse et d'un mosaicisme XX/XY.Arch. Fr. Pedriatr. 24:729 –739.

Dressler GR. 2006. The cellular basis of kidney development. Ann. Rev. Cell Dev. Biol . 22:509-529.

Dressler GR. 2009. Advances in early kidney specification, development and patterning. Development 136:3863-3874.

Durst KL, and Hiebert SW. 2004. Role of RUNX family members in transcriptional repression and gene silencing. Oncogene 23:4220-4224.

Dy P, Penzo-Mendez A, Wang H, Pedraza C, Macklin W, V L. 2008. The three SoxC proteins—Sox4, Sox11 and Sox12—exhibit overlapping expression patterns and molecular properties. Nucleic Acids Res 36:3101–3117.

Eurell JA, and Frappier BL 2006. Dellman's Textbook of Veterinary Histology, 6th ed., Blackwell Publishing. Urinary system, 212-213.

Fogo AB and Kon V. 2010. The glomerulus--a view from the inside--the endothelial cell. Int. J. Biochem. Cell Biol. 42:1388-1397.

Fogelgren B, Yang S, Sharp IC, Huckstep OJ, Ma W, Somponpun SJ, Carlson EC, Uyehara CF, Lozanoff S. 2009. Deficiency in Six2 during prenatal development is associated with reduced nephron number, chronic renal failure, and hypertension in br/+ adult mice. Am. J. Physiol. Renal Physiol. 296:F1166-F1178.

Frasier SD, Bashore RA, Mosier HD. 1964. Gonadoblastoma associated with pure gonadal dysgenesis in monozygotic twins. J. Pediatr. 64:740–5.

Fujita Y, Nishimura M, Taniwaki M, Abe T, Okuda T. 2001. Identification of an alternatively spliced form of the mouse AML1/RUNX1 gene transcript AML1c and its expression in early hematopoietic development. Biochem. Biophys. Res. Commun. 281:1248–1255.

Geijsen N, Uings IJ, Pals C, Armstrong J, McKinnon M, Raaijmakers JAM, Lammers JJ, Koenderman L, Coffer PJ. 2001. Cytokine-Specific transcriptional regulation through an IL-5Ra interacting protein. Science 293:1136-1138.

Genepaint.2003.
http://www.genepaint.org/cgi-bin/mgrqcgi94?APPNAME=genepaint&PRGNAME=analysis_viewer&ARGUMENTS=-AQ25250083349408,-AMH,-A564,-Asetview

Georgas K, Rumballe B, Valerius MT, Chiu HS, Thiagarajan RD, Lesieur E, Aronow BJ, Brunskill EW, Combes AN, Tang D, Taylor D, Grimmond SM, Potter SS, McMahon AP, Little MH. 2009. Analysis of early nephron patterning reveals a role for distal RV proliferation in fusion to the ureteric tip via a cap mesenchyme-derived connecting segment. Dev. Biol. 332:273-286.

Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GAP. 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 343:774–778.

Gilbert, SF. 2010. Developmental Biology. Vol 9th, (Sinauer Associates, Inc.)

Gray PA, Fu H, Luo P, Zhao Q, Yu J, Ferrari A, TenzenT, Yuk DI, Tsung EF, Cai Z, Alberta JA, Cheng LP, Liu Y, Stenman JM, Valerius MT, Billings N, Kim HA, Greenberg ME, McMahon AP, Rowitch DH, Stiles CD, Ma Q. 2004. Mouse brain organization revealed through direct genome-scale TF expression analysis. Science 306:2255-2257

Grosschedl R, Giese J, Pagel J. 1994. HMG domain proteins: Architectural elements in the assembly of nucleoprotein structures. Trends Genet. 10:94–100.

Grubb GR, Yun K, Williams BR, Eccles MR, Reeve AE. 1994. Expression of WT1 protein in fetal kidneys and Wilms tumors. Lab. Invest. 71:472– 479.

Guyton A, and Hall J. 2006. Textbook of medical physiology/11th ed. Elsevier Inc. 307-308

Hargrave M, Wright E, Kun J, Emery J, Cooper L, Koopman P. 1997. Expression of the SOX11 gene in mouse embryos suggests roles in neuronal maturation and

epitheliomesenchymal induction. Dev. Dyn. 210:79–86.

Hartwig S, Ho J, Pandey P, Macisaac K, Taglienti M, Xiang M, Alterovitz G, Ramoni M, Fraenkel E, Kreidberg JA. 2010. Genomic characterization of Wilms' tumor suppressor 1 targets in nephron progenitor cells during kidney development. Development 137:1189-1203.

Hattori T, Coustry F, Stephens S, Eberspaecher H, Takigawa M, Yasuda H, de Crombrugghe B. 2008. Transcriptional regulation of chondrogenesis by coactivator Tip60 via chromatin association with Sox9 and Sox5. Nucleic Acids Res. 36:3011-3024.

Hartkamp J and Roberts SG. 2008. The role of the Wilms' tumour-suppressor protein WT1 in apoptosis. Biochem Soc Trans 36:629-31.

Herzer U, Crocoll A, Barton D, Howells N, Englert C. 1999. The Wilms tumor suppressor gene WT1 is required for development of the spleen. Curr. Biol. 9:837–840.

Hino S, Kishida S, Michiue,T, Fukui A, Sakamoto I, Takada S, Asashima M, Kikuchi A. 2001. Inhibition of the Wnt signaling pathway by Idax, a novel Dvl-binding protein. Mol. Cell. Biol. 21:330-342.

Hosking BM, Wang SC, Chen SL, Penning S, Koopman P, Muscat GE. 2001. SOX18 directly interacts with MEF2C in endothelial cells. Biochem. Biophys. Res. Commun. 287:493–500.

Huang J, Arsenault M,Kann M, Lopez-Mendez C, Saleh M, Wadowska D, Taglienti M, Lefebvre V, Ho J, Miao Y, Sims D, Spears J, Lopez A, Wright G, Hartwig S. 2013**.** The transcription factor Sry-related HMG box-4 (SOX4) is required for normal renal development in vivo. Dev Dyn. 42:790-799.

Hueber PA, Fukuzawa,R, Elkares R, Chu L, Blumentkrantz M, He SJ. Anaka MR, Reeve AE, Eccles M, Jabado N, Iglesias DM, Goodyer PR. 2009. PAX3 is expressed in the stromal compartment of the developing kidney and in Wilms tumors with myogenic phenotype. Pediatr. Dev. Pathol. 12:347-354.

Hur W, Rhim H, Jung CK, Kim JD, Bae SH, Jang JW, Yang JM, Oh ST, Kim DG, Wang HJ, Lee SB, Yoon SK. 2010. SOX4 overexpression regulates the p53-mediated apoptosis in hepatocellular carcinoma: clinical implication and functional analysis *in vitro*. Carcinogenesis 31:1298-1307.

Iguchi H, Urashima Y, Inagaki Y, Ikeda Y, Okamura M, Tanaka T, Uchida A,

Yamamoto TT, Kodama T, Sakai J. 2007. SOX6 suppresses cyclin D1 promoter activity by interacting with beta-catenin and histone deacetylase 1, and its down-regulation induces pancreatic beta-cell proliferation. J. Biol. Chem. 282:19052-19061.

Joseph A, Yao H, Hinton BT. 2009. Development and morphogenesis of the Wolffian/epididymal duct, more twists and turns. Dev. Biol. 325:6-14.

Kamachi Y, Uchikawa M, Kondoh H. 2000. Pairing SOX off: with partners in the regulation of embryonic development. Trends Genet. 16:182–187.

Kamachi Y, Uchikawa M, Tanouchi A, Sekido R, Kondoh H. 2001. Pax6 and SOX2 form a co-DNA-binding partner complex that regulates initiation of lens development. Genes Dev. 15:1272-1286.

Kiefer JC. 2007. Back to Basics: SOX Genes. Dev. Dyn. 236:2356–2366.

Kim, DW, and Lassar AB. 2003. Smad-dependent recruitment of a histone deacetylase/Sin3A complex modulates the bone morphogenetic protein-dependent transcriptional repressor activity of Nkx3.2. Mol .Cell Biol. 23:8704-8717.

Kim EJ, Cho SW, Shin JO, Lee MJ, Kim KS, Jung HS. 2013. Ihh and Runx2/Runx3 signaling interact to coordinate early chondrogenesis: A mouse model. PLoS One 8:e55296.

Kim S, and Chung DH. 2006. Pediatric solid malignancies: Neuroblastoma and Wilms' tumor. Surg. Clin. North Am. 86:469, 87, xi.

Kitsiou-Tzeli S, Deligiorgi M, Malaktari-Skarantavou S, Vlachopoulos C, Megremis S, Fylaktou I, Traeger-Synodinos J, Kanaka-Gantenbein C, Stefanadis C, Kanavakis E. 2012. Sertoli cell tumor and gonadoblastoma in an untreated 29-year-old 46,XY phenotypic male with Frasier syndrome carrying a WT1 IVS9+4C>T mutation. Hormones (Athens) 11:361-7.

Klemm JD, Rould MA, Aurora R, Herr W, Pabo CO. 1994. Oct-1 POU domain-DNA interactions: cooperative binding of isolated sub-domains and effects of covalent linkage. Cell 77:21–32.

Kondoh H and Kamachi Y. 2010. SOX-partner code for cell specification: Regulatory target selection and underlying molecular mechanisms. Int. J. Biochem. Cell Biol. 42:391-399.

Koopman P. 1999. Sry and SOX9: mammalian testis determining genes. Cell. Mol. Life Sci .55:839–856.

Kormish JD, Sinner D, Zorn AM. 2010. Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease. Dev. Dyn. 239:56-68.

Kreidberg JA. 2010. WT1 and kidney progenitor cells. Organogenesis 6:61-70.

Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R. 1993. WT-1 is required for early kidney development. Cell 74:679 – 691.

Kuhlbrodt K, Herbarth B, Sock E, Enderich J, Hermans-Borgmeyer I, Wegner M. 1998a. Cooperative function of POU proteins and SOX proteins in glial cells. J. Biol. Chem. 273:16050–16057.

Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M. 1998b. SOX10, a novel transcriptional modulator in glial cells. J. Neurosci. 18:237–250.

Kwakowsky A, Schwirtlich M, Zhang Q, Eisenstat DD, Erdelyi F, Baranyi M, Katarova ZD, Szabo G. 2007. GAD isoforms exhibit distinct spatiotemporal expression patterns in the developing mouse lens: correlation with Dlx2 and Dlx5. Dev. Dyn. 236:3532-3544.

Latchman DS. 1999. POU family transcription factors in the nervous system. J. Cell Physiol. 179:126-133.

Laudet V, Stehelin D, Clevers H. 1993. Ancestry and diversity of the HMG box superfamily. Nucleic Acids Res. 21:2493–2501.

Leon TY, Ngan ES, Poon HC, So MT, Lui VC, Tam PK, Garcia-Barcelo MM. 2009. Transcriptional regulation of RET by Nkx2-1, Phox2b, Sox10, and Pax3. J. Pediatr. Surg. 44:1904-1912.

Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N,Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y. 2002. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 109:113–124.

Liao X, Wang Y, Wong CW. 2010. Troglitazone induces cytotoxicity in part by promoting the degradation of peroxisome proliferator-activated receptor y co-activator-1a protein. Br. J. Pharmacol. 161:771-781.

Little MH, and McMahon AP. 2012. Mammalian kidney development: Principles, progress, and projections. Cold Spring Harb Perspect Biol 4:10.1101/cshperspect.a008300.

Little S, Hanks S, King-Underwood L, Picton S, Cullinane C, Rapley E, Rahman N, Pritchard-Jones K. 2005. A WT1 exon 1 mutation in a child diagnosed with denys-drash syndrome. Pediatr Nephrol 20:81-85.

Liu Z, Hu X, Cai J, Liu B, Peng X, Wegner M, Qiu M. 2007. Induction of oligodendrocyte differentiation by Olig2 and Sox10: Evidence for reciprocal interactions and dosage-dependent mechanisms. Dev. Biol. 302:683-693.

Lodato MA, Ng CW, Wamstad JA, Cheng AW, Thai KK, Fraenkel E, Jaenisch R, Boyer LA. 2013. SOX2 co-occupies distal enhancer elements with distinct POU factors in ESCs and NPCs to specify cell state. PLoS Genet 9: Epub 2013 Feb 21

Majumdar A, Vainio S, Kispert A, McMahon J, McMahon AP. 2003. Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. Development 130:3175-3185.

Marfil V, Moya M, Pierreux CE, Castell JV, Lemaigre FP, Real FX, Bort R. 2010. Interaction between hhex and SOX13 modulates Wnt/TCF activity. J. Biol. Chem. 285:5726-5737.

Masuda T, and Esumi N. 2010. SOX9, through interaction with microphthalmia-associated transcription factor (MITF) and OTX2, regulates BEST1 expression in the retinal pigment epithelium. J. Biol. Chem. 285:26933-26944.

Melchionda F, Spreafico F, Ciceri S, Lima M, Collini P, Pession A, Massimino M, Radice P, Perotti D. 2013. A novel WT1 mutation in familial Wilms tumor. Pediatr Blood Cancer . Mar 28. doi: 10.1002/pbc.24539. [Epub ahead of print]

Melnikova IN, Yang Y, Gardner PD. 2000. Interactions between regulatory proteins that bind to the nicotinic receptor beta4 subunit gene promoter. Eur. J. Pharmacol. 393:75-83.

Modrell MS, Buckley D, Baker CV. 2011. Molecular analysis of neurogenic placode development in a basal ray-finned fish. Genesis 49:278-294.

Mrowka C, Schedl A. 2000. Wilms' tumor suppressor gene WT1: From structure to renal pathophysiologic features. J. Am. Soc. Nephrol. 11 Suppl. 16:S106-15.

Murakami A, Ishida S, Thurlow J, Revest JM, Dickson C. 2001. SOX6 binds CtBP2 to repress transcription from the fgf-3 promoter. Nucleic Acids Res 29:3347-3355.

Nachtigal MW, Hirokawa Y, Enyeart VanHouten DL, Flanagan JN, Hammer GD, Ingraham HA. 1998. Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell 93:445-454.

Nishimoto M, Fukushima A, Okuda A, Muramatsu M. 1999. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol. Cell. Biol. 19:5453-5465.

Okuda T, Nishimura M, Nakao M, Fujita Y. 2001. RUNX1/AML1: A central player in hematopoiesis. Int. J. Hematol. 74:252-257.

Pan X, Li H, Zhang P, Jin B, Man J, Tian L, Su G, Zhao J, Li W, Liu H, Gong W, Zhou T, Zhang X. 2006. Ubc9 interacts with SOX4 and represses its transcriptional activity. Biochem .Biophys. Res. Commun. 344:727-734.

Pandey PK, Udayakumar TS, Lin X, Sharma D, Shapiro PS, Fondell JD. 2005. Activation of TRAP/Mediator subunit TRAP220/Med1 is regulated by mitogen-activated protein kinase-dependent phosphorylation. Mol. Cell. Biol. 25:10695-10710

Panganiban G, Rubenstein JL. 2002. Developmental functions of the distal-less/Dlx homeobox genes. Development 129:4371-4386.

Pastore G, Znaor A, Spreafico F, Graf N, Pritchard-Jones K, and Steliarova-Foucher E. 2006. Malignant renal tumours incidence and survival in European children (1978-1997): report from the Automated Childhood Cancer Information System project. Eur. J. Cancer 42:2103-2114.

Park JS, Valerius MT, McMahon AP. 2007. Wnt/beta-catenin signaling regulates nephron induction during mouse kidney development. Development 134:2533-2539.

Pfaffl MW. 2004. Quantification strategies in real-time PCR. In: Bustin SA, editor. A-Z of Quantitative PCR. La Jolla, CA, USA: International University Line. p. 87-112.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26:509-515.

Pietila I, Vainio S. 2005. The embryonic aorta-gonad-mesonephros region as a generator of haematopoietic stem cells. Apmis 113:804–812.

Pole RJ, Qi BQ, Beasley SW. 2002. Patterns of apoptosis during degeneration of the pronephros and mesonephros. J. Urol. 167:269–271.

Qiu M, Bulfone A, Martinez S, Meneses JJ, Shimamura K, Pedersen RA, Rubenstein JL. 1995. Null mutation of dlx-2 results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. Genes Dev. 9:2523-2538.

Quaggin SE and Kreidberg JA. 2008. Development of the renal glomerulus: Good neighbors and good fences. Development 135:609-620.

Rauscher FJ. 1993. The WT1 wilms tumor gene product: A developmentally regulated transcription factor in the kidney that functions as a tumor suppressor. FASEB J. 7:896-903.

Reginensi A, Clarkson M, Neirynck Y, Lu B, Ohyama T, Groves AK, Sock E, Wegner M, Costantini F, Chaboissier MC, Schedl A. 2011. SOX9 controls epithelial branching by activating RET effector genes during kidney development. Hum. Mol. Genet. 20:1143-1153.

Rumballe B, Georgas K, Wilkinson L, Little M. 2010. Molecular anatomy of the kidney: What have we learned from gene expression and functional genomics? Pediatr. Nephrol. 25:1005-1016.

Sainio K. 2003. Development of the mesonephric kidney. The kidney. From normal development to congenital disease Vize, C. Woolf, A. S. Bard, J. B. L. London: Academic Press; pp. 75–86.

Sainio K, Hellstedt P, Kreidberg JA, Saxen L, Sariola H. 1997. Differential regulation of two sets of mesonephric tubules by WT-1. Development 124:1293–1299.

Sakurai N, Fujii T, Hashizume T, Sawai K. 2013. Effects of downregulating Oct-4 transcript by RNA interference on early development of porcine embryos. J. Reprod. Dev. Apr 29. [Epub ahead of print]

Schilham MW, Oosterwegel MA, Moerer P, Ya J, de Boer PA, van de Wetering M, Verbeek S, Lamers WH, Kruisbeek AM, Cumano A, Levers H. 1996. Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking SOX-4.

Nature 380:711–714.

Schroeder TM, Jensen ED, Westendorf JJ. 2005. Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. Birth Defects Res. C Embryo. Today 75:213–225.

Schumacher V, Thumfart J, Drechsler M, Essayie M, Royer-Pokora B, Querfeld U, Muller D. 2006. A novel WT1 missense mutation presenting with Denys-Drash syndrome and cortical atrophy. Nephrol. Dial. Transplant 21:518-521.

Sharma PM, Yang X, Bowman M, Roberts V, Sukumar S. 1992. Molecular cloning of rat Wilms' tumor complementary DNA and a study of messenger RNA expression in the urogenital system and the brain. Cancer Res. 52:6407-6412.

Smith MA, Seibel NL, Altekruse SF, Ries LA, Melbert DL, O'Leary M, Smith FO, Reaman GH. 2010. Outcomes for children and adolescents with cancer: Challenges for the twenty-first century. J. Clin. Oncol. 28:2625-2634.

Soullier S, Jay P, Poulat F, Vanacker JM, Berta P, Laudet V. 1999. Diversification pattern of the HMG and SOX family members during evolution. J. Mol. Evol. 48:517–527.

Stewenius Y, Jin Y, Ora I, de Kraker J, Bras J, Frigyesi A, Alumets J, Sandstedt B, Meeker AK, Gisselsson D. 2007. Defective chromosome segregation and telomere dysfunction in aggressive Wilms' tumors. Clin. Cancer Res. 13(22 Pt 1):6593-6602.

Stock DW, Ellies DL, Zhao Z, Ekker M, Ruddle FH, Weiss KM. 1996. The evolution of the vertebrate Dlx gene family. Proc. Natl. Acad. Sci. USA 93:10858-10863.

Taatjes DJ. 2010. The human Mediator complex: a versatile, genome-wide regulator of transcription. Trends Biochem. Sci. 35:315-322.

Tanaka S, Kamachi Y, Tanouchi A, Hamada H, Jing N, Kondoh H. 2004. Interplay of SOX and POU factors in regulation of the nestin gene in neural primordial cells. Mol Cell Biol 24(20):8834-46.

Thevenet L, Albrecht KH, Malki S, Berta P, Boizet-Bonhoure B, Poulat F. 2005. NHERF2/SIP-1 interacts with mouse SRY via a different mechanism than human SRY. J. Biol. Chem. 280:38625-38630.

Toka HR, Toka O, Hariri A, Nguyen HT. 2010. Congenital anomalies of kidney and urinary tract. Semin. Nephrol. 30:374-386.

Uwanogho D, Rex M, Cartwright EJ, Pearl G, Healy C, Scotting PJ. 1995. Embryonic expression of the chicken SOX2, SOX3 and SOX11 genes suggests an interactive role in neuronal development. Mech. Dev. 49:23–36.

Vetter MR, and Gibley CW. 1966. Morphogenesis and histochemistry of the developing mouse kidney. J. Morphol. 12:135–155.

Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. 1996. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc. Natl. Acad. Sci. 93:3444–3449.

Wessely O and Tran U. 2011. Xenopus pronephros development--past, present, and future. Pediatr. Nephrol. 26:1545-1551.

Wilson M, and Koopman P. 2002. Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. Curr. Opin. Genet. Dev. 12:441–446.

Wissmuller S, Kosian T, Wolf M, Finzsch M, Wegner M. 2006. The high-mobility-group domain of Sox proteins interacts with DNA-binding domains of many transcription factors. Nucleic Acids Res. 34:1735-1744.

Zhou G, Zheng Q, Engin F, Munivez E, Chen Y, Sebald E, Krakow D, Lee B. 2006. Dominance of SOX9 function over RUNX2 during skeletogenesis. Proc. Natl. Acad. Sci. USA 103:19004-19009.

Zhou R, Bonneaud N, Yuan CX, de Santa Barbara P, Boizet B, Schomber T, Scherer G, Roeder RG, Poulat F, Berta P. 2002. SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex. Nucleic Acids Res. 30:3245-3252

Zugor V, Zenker M, Schrott KM, Schott GE. 2006. Frasier syndrome: A rare syndrome with WT1 gene mutation in pediatric urology. Aktuelle Urol. 37:64-66.

Zhu Y, Li Y, Wei J, Liu X. 2012. The role of *Sox* genes in lung morphogenesis and cancer. Int. J. Mol. Sci. 13:15767-15783.

7. APPENDICES

7.1 APPENDIX A- RNA isolation protocol

Note:

1. All glassware used during RNA isolation was autoclaved and baked at 250^0C to ensure that it was RNase/ DNase free.
2. All required equipment that was not baked such as pipette tips, micro-centrifuge tube, collection tube and syringes were sterile.
3. Surface area where the isolation procedure was carried out was first cleaned with 70% ethanol, then with RNA Zap to ensure the surface was clean and free of RNase/DNase.
4. Samples being used during the RNA isolation were kept cold on ice to ensure no degradation of RNA occurred during the process.

Clean the homogenizer

1. Separate the homogenizer, spray with RNA Zap, wait 5-10 minutes.
2. Clean homogenizer with 70% ethanol prepared with UltraPure distilled (RNase/DNase free) water and then with autoclaved ddH_2O between different aged samples.

Homogenization of Samples

Note:

Transfer the needed kidneys to a culture tube containing 350 μl buffer RA1 and 3.5 μl beta-mercaptoethanol and put the tube on ice.

Technique

1. Place the culture tube in a beaker containing ice water to keep the tube cold.
2. Homogenize until no obvious tissue exists.
3. Transfer the amount of cells to the 0.9 mm needle (20 – 23.5 gauge).

4. Transfer to the RNA spin Mini Filter units in the collection tube.

RNA isolation

Technique

1. Centrifuge the RNA spin Mini Filter units with the collection tube for 1 min at 14,000 rpm, 4^0C in the microcentrifuge
2. Discard the RNA spin Mini Filter. Transfer filtrate to a new 1.5 ml microcentrifuge tube.
3. Add 350 μl 70% ethanol to the homogenized lysate and mix by vortexing. 2 x 5 sec.
4. Add 350 μl 70% ethanol again to the homogenized lysate and mix by vortexing. 2 x 5 sec.
5. Transfer half of the lysate to the RNAspin Mini column placed in a 2 ml microcentrifuge tube. Pipette lystate up and down 2-3 times. Centrifuge for 30 s at 11,000 rpm, 4^0C. Discard the flow through, and return the column to the collection tube.
6. Transfer the rest of the lysate to the RNAspin Mini column placed in a 2 ml microcentrifuge tube (Be sure to load all of the precipitate onto the column). Pipette lysate up and down 2-3 times. Centrifuge for 30 sec at 11,000 rpm, 4^0C. Pace the column in a new collection tube.
7. Add 350 μl MDB and centrifuge at 14,000rpm for 1 min, 4^0C. Discard the flow-through and return the column to the collection tube.
8. Add 10 μl reconstituted DNase I to 90 μl DNase reaction buffer. Mix by flicking the tube. Apply 95 μl of DNase reaction mixture directly onto the center of the silica membrane of the column. Incubate at room temperature for 15 min.
9. Add 200 μl buffer RA2 to the RNAspin Mini column. Centrifuge for 1 min at 14,000 rpm, 4^0C.Place the column into a new collection tube.
10. Add 600 μl buffer RA3 to the RNAspin Mini column. Centrifuge for 1 min at 14,000 rpm, 4^0C. Discard flow-through and place the column back.
11. Add 250 μl buffer RA3 to the RNAspin Mini column. Centrifuge for 2 min at 14,000 rpm,4^0C. Place the column into a nuclease-free 1.5 ml microcentrifuge tube.
12. Elute the RNA in 100 μl RNase free H_20 and set for 2 mins. Centrifuge for 1 min at 14,000 rpm,4^0C.
13. Elute the RNA again in 100 μl RNase free H_20 and set for 2 mins. Centrifuge for 1 min at 14,000 rpm,4^0C.
14. Transfer the microcentrifuge tube with the sample to the -80^0C.

7.2 APPENDIX B- cDNA synthesis protocol

cDNA synthesis is carried out using the qScript^{tm} cDNA synthesis kit. The kit uses 20 μl synthesis volume and 1 μl of sample. The 1 μg sample is determined by dividing 1000 ng by the ng/μl reading from Nanodrop 1000.

Components:

1. 5X Reaction Mix 4μl
2. Reverse Transcriptase 1μl
3. Nuclease free water 15-X
4. mRNA X X=1000 ng/Concentration per μl of RNA sample

   Example: The concentration of E13.5 kidney RNA determined by Nanodrop is 285 ng/μl. Therefore, the amount of RNA added to cDNA synthesis will be 1000 ng/ (285 ng/μl) = 3.5 μl of RNA solution.

For the negative control (Minus-RT Control: Without the Reverse Transcriptase in the reaction in order to make sure there is no genomic DNA contamination):

1. 5X Reaction Mix 4 μl
2. Nuclease free water 16-X

3. mRNA: X X=1000 ng/Concentration per μl of RNA sample

Technique

1. Combine the reagents in a 0.2 ml thin walled PCR tube or micro-centrifuge tube and placed over ice. Reverse transcriptase is added in the beginning to ensure it has been added and not lost in the preparation.
2. Vortex gently and then centrifuge for 10 seconds to collect content to the bottom of the tube.
3. Place the tube in the thermal cycler which is programmed as follow:
1. Forever: 25°C
2. 1 Cycle: 25°C, 5 minutes
3. 1 Cycle: 42°C, 30 minutes

4. 1 Cycle: 85°C, 5 minutes
5. Forever: 4°C
6. Initiate run
7. After cDNA synthesis, the cDNA was diluted 20X and stored at -20°C.

Notes: All the components prior to and after cDNA synthesis should be kept on ice to maintain integrity of the sample and prevent any degradation.

7.3 APPENDIX C- List of primer information and sequences for the potential SOXC signaling partners

Table 6. List of primer information and sequences for the potential SoxC signalling partners. Primer pairs were designed to ensure that primers cross at least one intron sequence to reduce risk of co-amplified genomic DNA. Primer pairs that did not cross intron-exon boundaries included a negative (-RT) control.

Name	Sequences	Accession Nos.	Amplicon Size	Intron-exon boundary
Cbp forward	AGGTGTTTCAC AGGGTCAGG	NM_001025432	113	Yes
Cbp reverse	GGTGCAATGGT GACTGTGTC			
Ctbp1 (p1) forward	GTGCCCTGATG TACCATACCA	NM_013502	108	No
Ctbp1 (p1) reverse	CGGGTTTGACA ATATCGACATC A			
Ctbp2 forward	GGCAGCGATTG GACAGGATTT	NM_ 001170744	121	Yes
Ctbp2 reverse	AGGATGGGCAT CTCCACAGT			
Cxxc4 forward	ACAGACAGTG CGTTTCAAATT GC	NM_ 001004367	161	NO
Cxxc4 reverse	CCACAGTTGAT GAGCCTCTTG			
Cxxc5 forward	CAGGAGGAAC AGACAAAAGT ACC	NM_133687	126	NO
Cxxc5 reverse	GGCTCTTGTTG AGGGGTTCAC			
Dlx1 forward	ATGCCAGAAAG TCTCAACAGC	NM_010053	118	NO
Dlx1 reverse	AACAGTGCATG GAGTAGTGCC			
Dlx2 forward	GGCTCCTACCA GTACCACG	NM_004405	133	NO
Dlx2 reverse	CGTTGTTGACC			

	GGAGACGAA			
Dlx3 forward	CACTGACCTGG GCTATTACAGC	NM_010055	103	NO
Dlx3 reverse	GAGATTGAACT GGTGGTGGTAG			
Dlx4 forward	GCAAGCCCAG AACCATCTACT	NM_007867	117	NO
Dlx4 reverse	TGAGTCCGAGT TGTGCTGC			
Dlx5 forward	GTCCCAAGCAT CCGATCCG	NM_010056	79	NO
Dlx5 reverse	GCGATTCCTGA GACGGGTG			
Dlx6 forward	AAAACGACAG TGATCGAAAAC GG	NM_010057	123	NO
Dlx6 reverse	AGTCTGCTGAA AGCGATGGTT			
Egr1 forward	CCACAACAAC AGGGAGACCT	NM_007913	124	NO
Egr1 reverse	ACTGAGTGGCG AAGGCTTTA			
Egr2(old) forward	GCCAAGGCCGT AGACAAAATC	NM_010118	154	NO
Egr2 (old)reverse	CCACTCCGTTC ATCTGGTCA			
Egr3forward	CCGGTGACCAT GAGCAGTTT	NM_018781	122	NO
Egr3 reverse	TAATGGGCTAC CGAGTCGCT			
Ep300 forward	TAAACATGGCT CCACAACCA	NM_177821	106	NO
Ep300 reverse	GACCTGAGAGT CCGCAAAAG			
Hdac1 forward	AGTCTGTTACT ACTACGACGGG	NM_ 008228	101	NO
Hdac1 reverse	TGAGCAGCAAA TTGTGAGTCAT			
Hdac10 forward	GTGCGAAATTG AGTGCCCAG	NM_199198	102	YES
Hdac10 reverse	GATGCCTCACA			

	AGCTGACAAA			
Hdac11 forward	ATCTACCCTGG GGATCGCTTT	NM_144919	110	YES
Hdac11 reverse	CTCCTGACATT CCTCTCCACC			
Hdac2(p1) forward	GGAGGAGGCT ACACAATCCG	NM_008229	173	YES
Hdac2(p1) reverse	TCTGGAGTGTT CTGGTTTGTCA			
Hdac3(p2) forward	GGTTCAGGAA GCCTTCTACCT	NM_010411	112	YES
Hdac3(p2) reverse	ACTCTCTGCTC CAACTTCATAC A			
Hdac5 forward	AGCACCGAGGT AAAGCTGAG	NM_001077696	91	NO
Hdac5 reverse	GCTGTGGGAG GGAATGGTT			
Hdac6 forward	TCCACCGGCCA AGATTCTTC	NM_001130416	109	YES
Hdac6 reverse	CAGCACACTTC TTTCCACCAC			
Hdac7 forward	TTCCCTTGCGT AAAACAGTGT	NM_019572	87	NO
Hdac7 reverse	GCAGGGGATTC TTGCGTCT			
Hdac8 forward	ACTATTGCCGG AGATCCAATGT	NM_027382	107	NO
Hdac8 reverse	CCTCCTAAAAT CAGAGTTGCCA G			
Hdac9 forward	GCGGTCCAGGT TAAAACAGAA	NM_024124	108	NO
Hdac9 reverse	GCCACCTCAAA CACTCGCTT			
Hhex forward	CGGACGGTGA ACGACTACAC	NM_008245	150	YES
Hhex reverse	CTTCTCCAGCT CGACGGTC			
Hnrnpk forward	CAGCTCCCGCT CGAATCTG	NM_025279	210	Yes

Hnrnpk reverse	TCTGTCAGTAG AGTGAGGGCA			
Hoxa10 forward	CACCACCCACT CTGGTTTG	NM_008263	161	YES
Hoxa10 reverse	TGCATTTTCGC CTTTGGAACT			
Hoxa2 forward	TACGAATTTGA GCGAGAGATTG G	NM_010451	116	NO
Hoxa2 reverse	GTCGAGGTCTT GATTGATGAAC T			
Hoxa4 forward	GAAAGCACAA ACTCACAGCCC	NM_008265	169	NO
Hoxa4 reverse	TGTCTCGGGTT TACTTAGGGAA G			
Hoxa6 forward	CGGCCAGGACT CCTTCTTG	NM_010454	143	NO
Hoxa6 reverse	CCGAGTTGGAC TGTTGGTAAAA			
Hoxb4 forward	CGTGAGCACG GTAAACCCC	NM_010459	231	NO
Hoxb4 reverse	GTGTTGGGCAA CTTGTGGTC			
Hoxd4 forward	AAGTACGTGGA CCCCAAGTTT	NM_010469	245	NO
Hoxd4 reverse	GGGGCACCGTA ATGACTTCC			
Maf1forward	ATTGCCACCCT CAATGAGTC	NM_001164607	107	YES
Maf1 reverse	TTGACTGCATT TACCACCCA			
Med1 forward	GGCACCCCTGT TCGAGATTC	NM_001080118	262	NO
Med1 reverse	CCCCACTCTGA CTACTTTCATC A			
Med12 forward	CAGAGTGCTAT TAACACCTGGT T	NM_177855	179	YES

Med12 reverse	GCTGCATAGTA GGCACAAGTCA T			
Med15 forward	CGCCGGTTTGA GGAGGATG	NM_ 033609	128	NO
Med15 reverse	AGGTGGACAGT ACCGTTGTTG			
Med17 forward	ATGGCACCGAG ACGTACCT	NM_144933	237	YES
Med17 reverse	GGGCACTTCGC AAATTGTTCC			
Med18 forward	CACTGGGGGC ACCATTAACAT	NM_026039	180	YES
Med18 reverse	CTTAGGACGAA CGGGCTGG			
Med21 forward	GCTGTGAACTC GCTTGCAGA	NM_025315	122	YES
Med21 reverse	GTAGGATTGGC TGGTTGATCTT T			
Med23 forward	TTAAAGGGCGA GACCATCTGA	NM_001166416	73	NO
Med23 reverse	GCGAGGGCATT TTTCTGAATAC T			
Med25 forward	GTGGTGTTTGT GATCGAGGGG	NM_029365	215	YES
Med25 reverse	CTGGTAGGTGC GTGACATTGT			
Med27 forward	GATCGCACGAT TGTGAAGGG	NM_026896	150	YES
Med27 reverse	TGGCATCTGGG GTAACTGATAG			
Med28 forward	GCCCCGAGACC ATCTAACAG	NM_025895	210	YES
Med28 reverse	CTGGACAGATA ACTGCAACCTT			
Med29 forward	GCAGCAGCAG GACTTCGAT	NM_026042	195	YES
Med29 reverse	TCACAGAGTGC GTAAAACTCC			

Med30 forward	GCTGGTCCACC TCGTTTTG	NM_027212	124	YES
Med30 reverse	TGTCCCAGATG AGATTCCGTAA			
Med4 forward	CTCGGAGGCCA TACCCAAC	NM_026119	130	NO
Med4 reverse	ACATCTGGCAG TCTACCTGCT			
Med6 forward	TCTCCTGGGTT GACAGCTCTT	NM_27213	112	NO
Med6 reverse	ACCACCTCATT ATTACACGTCC T			
Mef-2a forward	CAGGTGGTGGC AGTCTTGG	NM_013597	132	YES
Mef-2a reverse	TGCTTATCCTTT GGGCATTCAA			
Mef-2b forward	AGGCTCCACAG ATTGCAGC	NM_001045484	82	YES
Mef-2b reverse	TACAGCGTCCC TCGTTGGT			
Mef-2c forward	TGCTGGTCTCA CCTGGTAAC	NM_025282	148	YES
Mef-2c reverse	ATCCTTTGATT CACTGATGGCA T			
Mef-2d forward	CGAGATCGCGC TCATCATCTT	NM_133665	164	YES
Mef-2d reverse	AGCCGTTGAAA CCCTTCTTCC			
Myst1 forward	ACGAGGCGATC ACCAAAGTG	NM_026370	166	NO
Myst1 reverse	AAGCGGTAGCT CTTCTCGAAC			
Myst2 forward	ATGCCGCGAAG GAAGAGAAAT	NM_001195004	164	NO
Myst2 reverse	TCTTGGGAACT CTGGCTTAGC			
Myst3 forward	CCTCGTGCATT GGCTGTTC	NM_001081149	106	NO
Myst3 reverse	TCATGGCATTC			

	AAGGTGTTCAT			
Myst4 (p2) forward	TGAGGAAAGC ATTCCCACATC	NM_017479	178	YES
Myst4 (p2) reverse	ACACTTGTCTG CACTTTAGAGG			
Nherf-2 forward	CACCTGCATGG CGAGAAAG	NM_001130012	128	No
Nherf-2 reverse	TCTACGTTGAC GCCGTTGAC			
Olig1 forward	TCTTCCACCGC ATCCCTTCT	NM_016968	226	NO
Olig1 reverse	CCGAGTAGGGT AGGATAACTTC G			
Olig2(p3) forward	GGCGGTGGCTT CAAGTCAT	NM_016967	99	NO
Olig2(p3) reverse	GGGCTCAGTCA TCTGCTTCT			
Olig3 forward	CAGGAGAGTC GTCTGAACTCG	NM_053008	181	NO
Olig3 reverse	GTTCGCGTCCG TTGATCTT			
Otx1 forward	ACTGACCCTTC GCCAGTCTA	NM_011023	124	NO
Otx1 reverse	AACATTGGACC TGGAACTCG			
Otx2 forward	CAGAGGTGATC CGGTGTTTT	NM_144841	89	NO
Otx2 reverse	CCTGTTGGTCA TGAAACGTG			
P53 forward	CTCTCCCCCGC AAAAGAAAAA	NM_001130012	84	Yes
P53 reverse	CGGAACATCTC GAAGCGTTTA			
P63(trp63) forward	CCCAAGTGGTG CCTCTACC	NM_011641	113	No
P63(trp63) reverse	CGTCACGGGGT GGAAAAGA			
P73 forward	GCACCTACTTT GACCTCCCC	NM_001126330	121	Yes
P73 reverse	GCACTGCTGAG			

	CAAATTGAAC			
Pax2(p1) forward	AAGCCCGGAG TGATTGGTG	NM_011037	101	NO
Pax2(p1) reverse	CAGGCGAACAT AGTCGGGTT			
Pax3 forward	GGGCAGAATTA CCCACGCA	NM_008781	177	YES
Pax3 reverse	CTGGCGAGAAA TGACGCAA			
Pax4 forward	GACTCAACTCA GATCACCAGC	NM_001159926	119	YES
Pax4 reverse	GGGAGAAGATA GTCCGATTCCT			
Pax5 forward	CCATCAGGACA GGACATGGAG	NM_008782	101	NO
Pax5 reverse	GGCAAGTTCCA CTATCCTTTGG			
Pax6 forward	AGGGCAATCGG AGGGAGTAA	NM_001244201	186	YES
Pax6 reverse	CAGGTTGCGAA GAACTCTGTTT			
Pax7 forward	TCTCCAAGATT CTGTGCCGAT	NM_011039	132	YES
Pax7 reverse	CGGGGTTCTCT CTCTTATACTCC			
Pax8(p1) forward	ATGCCTCACAA CTCGATCAGA	NM_011040	104	YES
Pax8(p1) reverse	ACAATGCGTTG ACGTACAACTT			
Pgc1a forward	AATGCAGCGGT CTTAGCACT	NM_008904	121	YES
Pgc1a reverse	TGTTGACAAAT GCTCTTCGC			
Pgc1b forward	CCGAGCTCTTC CAGATTGAC	NM_133249	116	YES
Pgc1b reverse	TTCATCCAGTT CTGGGAAGG			
Pou1f1(pit1) forward	ATGAGTTGCCA ATCTTTCACCT C	NM_008849	143	No
Pou1f1(pit1)	GCTGTGGACAT			

reverse	CACGTTGGT			
Pou2f1(oct1) forward	CCCAACTCGCA CAATAGCAG	NM_198933	69	NO
Pou2f1(oct1) reverse	ATTCGCTTTGG TGTTGACTGG			
Pou2f2 (new)forward	CTCGCACCTTC AAGCAACG	NM_011138	115	YES
Pou2f2 (new)reverse	TCGAAGCGGG AAATGGTCG			
Pou2f3(oct11) forward	CAGAGACGCAT TAAGCTAGGC	NM_011139	120	YES
Pou2f3(oct11) reverse	GCGAGATGGTA GTCTGGCT			
Pou3f1(oct6) forward	TCGAGGTGGGT GTCAAAGG	NM_011141	205	NO
Pou3f1(oct6) reverse	GGCGCATAAAC GTCGTCCA			
Pou3f2(brn2) forward	CGGATCAAACT CGGATTTACTC A	NM_008899	240	NO
Pou3f2(brn2) reverse	GGAGGTCCGCT TTTTCCGT			
Pou3f3(brn1) forward	AGCAGTTCGCT AAGCAGTTCA	NM_008900	123	NO
Pou3f3(brn1) reverse	CGAAGCGGCA GATAGTGGTC			
Pou3f4(brn4) forward	CTGCCTCGAAT CCCTACAGC	NM_008901	122	NO
Pou3f4(brn4) reverse	CTGCAAGTAGT CACTTTGGAGA A			
Pou4f1(brn3a) forward	CGCGCAGCGTG AGAAAATG	NM_011143	123	No
Pou4f1(brn3a) reverse	CGGGGTTGTAC GGCAAAATAG			
Pou4f2(brn3b) forward	TGGACATCGTC TCCCAGAGTA	NM_138944	91	No
Pou4f2(brn3b) reverse	GTGTTCATGGT GTGGTAAGTGG			
Pou4f3(brn3c) forward	CGACGCCACCT ACCATACC	NM_138945	114	No

Pou4f3(brn3c) reverse	CCCTGATGTAC CGCGTGAT			
Pou5f1(oct3,4) forward	AGTTGGCGTGG AGACTTTGC	NM_013633	160	No
Pou5f1(oct3,4) reverse	CAGGGCTTTCA TGTCCTGG			
Runx1 forward	GCAGGCAACG ATGAAAACTAC T	NM_009821	161	YES
Runx1 reverse	GCAACTTGTGG CGGATTTGTA			
Runx2 forward	ATGCTTCATTC GCCTCACAAA	NM_001145920	146	NO
Runx2 reverse	GCACTCACTGA CTCGGTTGG			
Runx3 forward	CAGGTTCAACG ACCTTCGATT	NM_019732	103	YES
Runx3 reverse	GTGGTAGGTAG CCACTTGGG			
Sf1 forward	ACCTGTAGCAT CGAGTGTCTT	NM_011750	151	NO
Sf1 reverse	CTGGAAGGGTC ACCAATGGG			
Smad1 forward	TCAATCCTTTT GTGTGGGGT	NM_008539	94	NO
Smad1 reverse	GATGATTCAAC GTGGGCTCT			
Smad2 forward	TCCCATTCCTG TTCTGGTTC	NM_010754	115	NO
Smad2 reverse	ACTGCCCACAC AAACCTTTC			
Smad3 forward	GCCTTTGTCTT CAGCACTCC	NM_016769	116	NO
Smad3 reverse	AGGCCAGCAC AGGACTCTAA			
Smad4 forward	GAGGAGATCGC TTTTGCTTG	NM_008540	112	YES
Smad4 reverse	CCAAACGTCAC CTTCACCTT			
Smad5 forward	TGCCTTTATGG GGAGTGAAG	NM_001164041	93	NO

Smad5 reverse	CCACCCAAGTC CACAGAACT			
Smad6 forward	ACAAGCCACTG GATCTGTCC	NM_008542	103	YES
Smad6 reverse	GACATGCTGGC ATCTGAGAA			
Smad7 forward	CGAGGAGGTG GTGTGTTTTT	NM_001042660	120	NO
Smad7 reverse	GAAGACTTGA GGGAGGGGAC			
Smad9 forward	AATGAATTTTC CCCCTTTGG	NM_019483	114	NO
Smad9 reverse	TTTGCTTGTCA GCTTCATGG			
Tip60(kat5) forward	TCCCGGTCCAG ATCACACTC	NM_001126330	130	Yes
Tip60(kat5) reverse	ACCTTCCGTTT CGTTGAGCG			
Ube2a forward	ATCCTAACGTC TATGCAGATGG T	NM_019668	187	Yes
Ube2a reverse	CGCTTTTCATAT TCCCGCTTGTT			
Ube2b forward	GAACCGAATCC AAACAGTCCA	NM_009458	100	Yes
Ube2b reverse	TTTGCTCAACA ATGGCCGAAA			
Ube2c forward	CTCCGCCTTCC CTGAGTCA	NM_026785	132	Yes
Ube2c reverse	GGTGCGTTGTA AGGGTAGCC			
Ube2d1 forward	CCCGTGGGAGA TGACTTGTTC	NM_145420	110	Yes
Ube2d1 reverse	GGATAGTCTGT CGGAAAGTGG A			
Ube2d2 forward	ACAAGGAATTG AATGACCTGGC	NM_019912	123	Yes
Ube2d2 reverse	CACCCTGATAG GGGCTGTC			
Ube2d3 forward	AACTTAGTGAT	NM_025356	106	Yes

	TTGGCCCGTG			
Ube2d3 reverse	GGGCTGTCATT AGGTCCCATAA			
Ube2f forward	CCAGATGAGGG TTACTACCAGG	NM_026454	110	Yes
Ube2f reverse	GGGTGCCAGAT TTTAGTCAAGC			
Ube2g1 forward	CTGGCAGAACT CAACAAAAATC C	NM_025985	137	Yes
Ube2g1 reverse	AGATGAGCCTT AAAAACACCA CC			
Ube2h forward	GACACGGACGT AGTCAAGCTC	NM_001169577	135	Yes
Ube2h reverse	GTCCACTCTCA CTTTCCACAC			
Ube2i forward	GGAGGAAGGA CCACCCTTTTG	NM_001177609	90	Yes
Ube2i reverse	GGATAGCGCAC TCCCAGTT			
Ube2j1 forward	ATGGAGACCCG CTACAACCT	NM_019586	100	Yes
Ube2j1 reverse	GAGGCTGAGC GTGGTAATGAT			
Ube2j2 forward	CAGAGCCCCTC CCTTCAAATA	NM_021402	179	Yes
Ube2j2 reverse	CAGCCTTGTGT TGCACTTAAAT C			
Ube2k forward	CAGCGAATCAA GCGGGAGTT	NM_016786	125	Yes
Ube2k reverse	AGGTCCTGCTA TTTCTCCTCTT			
Ybx1 forward	CAGACCGTAAC CATTATAGACG C	NM_011732	102	NO
Ybx 1 reverse	ATCCCTCGTTC TTTTCCCCAC			

7.4 APPENDIX D- RT-PCR conditions

Table 7. RT-PCR condition for all candidate partners: amount of primer used, denaturing temperature, annealing temperature, extension temperature, cycle number and betaine if required

Name	Primer (μl)	Denaturing Temp. (°C)	Annealing Temp. (°C)	Extension Temp. (°C)	Cycle Number	Adding Betaine
Col2a1	2.5	95	60	45	28	YES
Cbp	2.5	95	60	45	30	No
Ctbp1	2.5	95	60	45	45	No
Ctbp2	2.5	95	60	45	30	No
Cxxc4	2.5	95	55	45	38	No
Cxxc5	2.5	95	55	45	45	No
Dlx1	2.5	95	55	45	38	No
Dlx2	2.5	95	55	45	38	YES
Dlx3	2.5	95	55	45	38	No
Dlx4	2.5	95	55	45	38	No
Dlx5	1.5	95	65	45	38	No
Dlx6	2.5	95	55	45	45	No
Egr1	2.5	95	55	45	30	YES
Egr2	1.5	95	65	45	38	No
Egr3	2.5	95	60	45	38	YES
Ep300	2.5	95	55	45	30	No
Hdac1	2.5	95	55	45	30	No
Hdac10	2.5	95	55	45	30	No
Hdac11	2.5	95	55	45	45	No
Hdac2	2.5	95	55	45	30	No
Hdac3	2.5	95	55	45	30	No
Hdac5	2.5	95	55	45	30	No
Hdac6	2.5	95	55	45	30	No
Hdac7	2.5	95	55	45	30	No
Hdac8	2.5	95	55	45	30	No
Hdac9	2.5	95	55	45	30	No
Hhex	2.5	95	55	45	30	YES

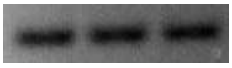
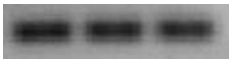
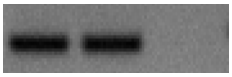
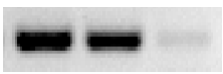
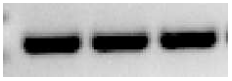
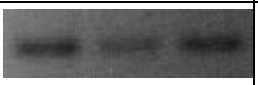
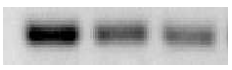
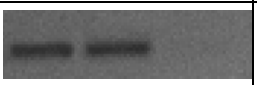
Hnrnpk	2.5	95	55	45	30	No
Hoxa10	2.5	95	55	45	45	No
Hoxa2	2.5	95	55	45	45	No
Hoxa4	1.5	95	65	45	38	No
Hoxa6	2.5	95	55	45	38	No
Hoxb4	2.5	95	55	45	30	No
Hoxd4	2.5	95	55	45	38	No
Maf1	2.5	95	55	45	30	No
Med1	2.5	95	55	45	30	No
Med12	2.5	95	60	45	30	No
Med15	2.5	95	55	45	30	No
Med17	2.5	95	55	45	30	No
Med18	2.5	95	55	45	30	No
Med21	2.5	95	55	45	30	No
Med23	2.5	95	55	45	30	No
Med25	2.5	95	60	45	30	No
Med27	2.5	95	55	45	30	No
Med28	2.5	95	55	45	30	No
Med29	2.5	95	55	45	30	No
Med30	2.5	95	55	45	38	No
Med4	2.5	95	55	45	30	No
Med6	2.5	95	55	45	30	No
Mef-2a	2.5	95	55	45	30	YES
Mef-2b	2.5	95	55	45	45	YES
Mef-2c	2.5	95	55	45	45	No
Mef-2d	2.5	95	55	45	30	No
Myst1	2.5	95	55	45	30	YES
Myst2	2.5	95	55	45	30	NO
Myst3	2.5	95	55	45	30	NO
Myst4	2.5	95	60	45	30	NO
Nherf-2	2.5	95	55	45	45	NO
Olig1	2.5	95	55	45	38	NO
Olig2	2.5	95	60	45	38	YES
Olig3	2.5	95	55	45	45	YES
Otx1	2.5	95	55	45	45	YES
Otx2	1.5	95	60	45	45	YES
P53	2.5	95	55	45	30	NO
P63	1.5	95	65	45	38	NO

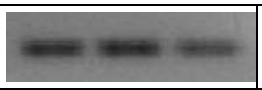
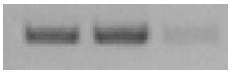
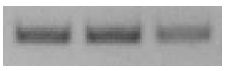
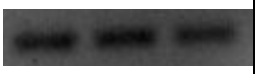
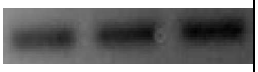
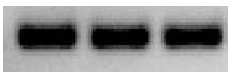
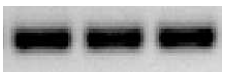
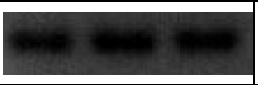
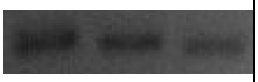
P73	1.5	95	65	45	38	NO
Pax2	2.5	95	55	45	30	NO
Pax3	2.5	95	55	45	38	NO
Pax4	2.5	95	60	45	38	YES
Pax5	2.5	95	55	45	45	YES
Pax6	2.5	95	55	45	45	NO
Pax7	2.5	95	55	45	38	NO
Pax8	2.5	95	55	45	30	NO
Pgc1a	2.5	95	55	45	30	NO
Pgc1b	2.5	95	60	45	38	NO
Pou 1f1	2.5	95	55	45	38	NO
Pou2F 1	2.5	95	55	45	30	NO
Pou2F 2	2.5	95	60	45	38	YES
Pou2f3	1.5	95	55	45	40	YES
Pou3f1	2.5	95	55	45	38	NO
Pou3F 2	1.5	95	55	45	40	YES
Pou3F 3	2.5	95	55	45	30	NO
Pou3f4	2.5	95	55	45	45	YES
Pou4f1	2.5	95	55	45	38	NO
Pou4f2	2.5	95	60	45	45	YES
Pou4f3	2.5	95	60	45	45	YES
Pou5f1	1.5	95	55	45	45	YES
Runx1	2.5	95	55	45	45	NO
Runx2	2.5	95	55	45	45	NO
Runx3	2.5	95	55	45	45	NO
Sf1	2.5	95	55	45	30	NO
Smad1	2.5	95	55	45	30	NO
Smad2	2.5	95	55	45	30	NO
Smad3	2.5	95	55	45	30	NO
Smad4	2.5	95	55	45	30	NO
Smad5	2.5	95	60	45	38	NO
Smad6	2.5	95	55	45	30	NO
Smad7	1.5	95	60	45	38	YES
Smad9	2.5	95	55	45	45	NO
Tip60	2.5	95	55	45	45	NO
Ube2a	2.5	95	55	45	30	NO

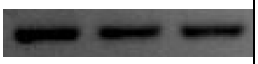
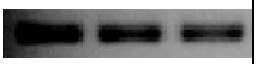
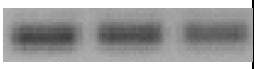
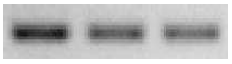
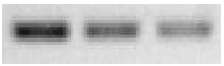
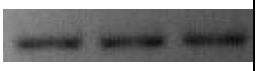
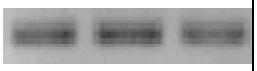
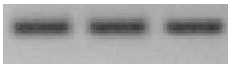
Ube2b	2.5	95	55	45	30	NO
Ube2c	2.5	95	55	45	30	NO
Ube2d1	2.5	95	55	45	30	NO
Ube2d2	2.5	95	55	45	30	NO
Ube2d3	2.5	95	55	45	30	NO
Ube2f	2.5	95	55	45	30	NO
Ube2g1	2.5	95	55	45	30	NO
Ube2h	2.5	95	55	45	30	NO
Ube2i	2.5	95	55	45	26	NO
Ube2j1	2.5	95	55	45	30	NO
Ube2j2	2.5	95	55	45	30	NO
Ube2k	2.5	95	55	45	30	NO
Yb1	2.5	95	55	45	30	NO

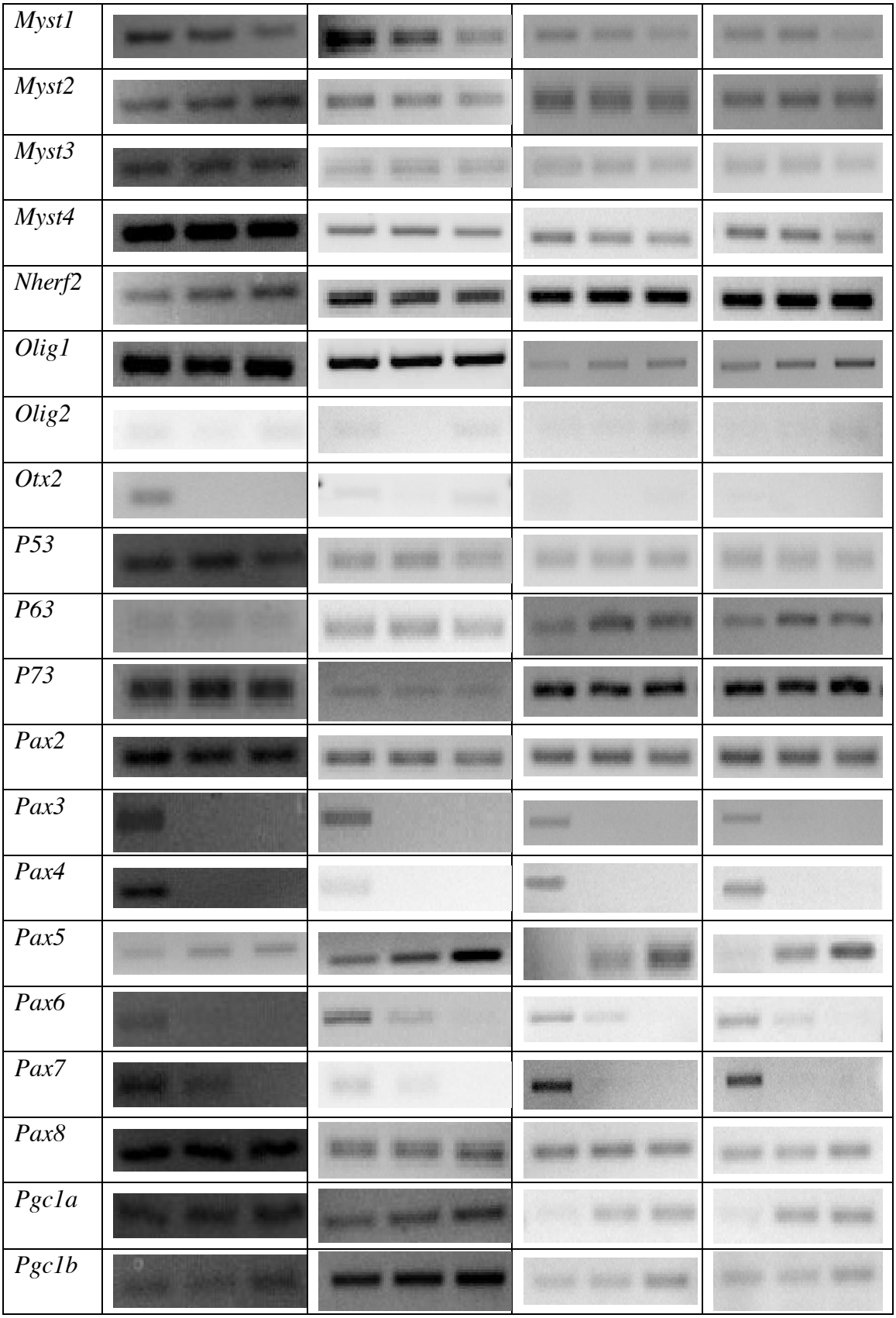
7.5 APPENDIX E-Expressions of all candidate partner transcripts of SoxC transcription factors in murine kidney tissue at E13.5, E18.5 and P21

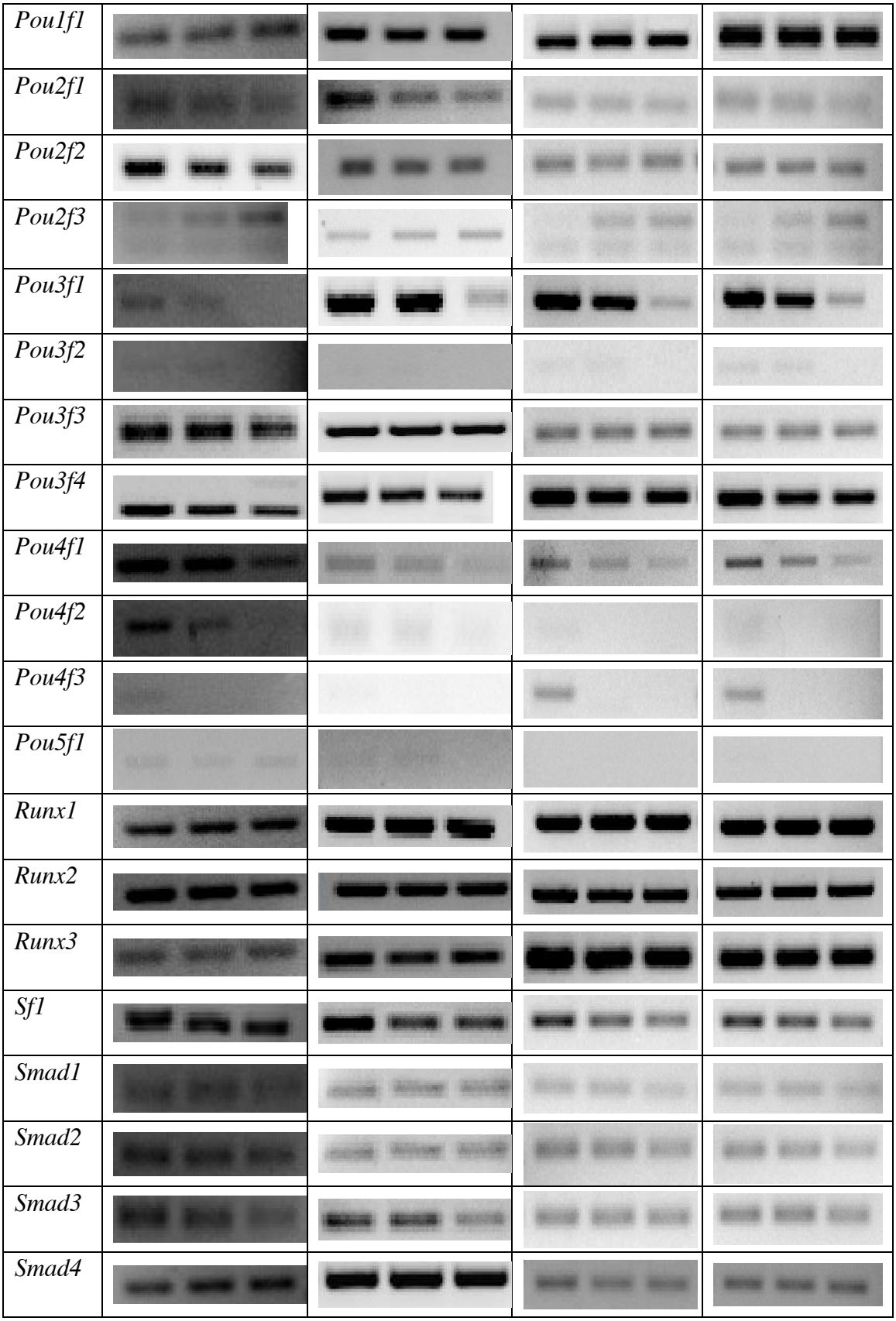
Figure 16. Expressions of all candidate partner transcripts of SoxC transcription factors in murine kidney tissue at E13.5, E18.5 and P21. N=4. Biological replicate 1 (Bio 1), Biological replicate 2(Bio 2), Biological replicate 3(Bio 3), Biological replicate 4(Bio 4).

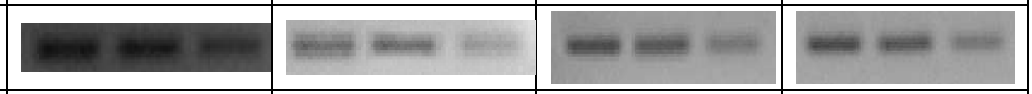
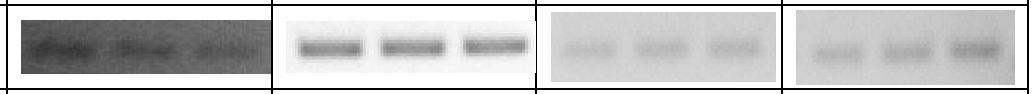
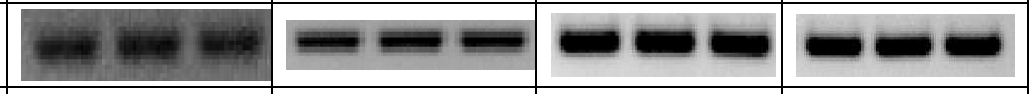
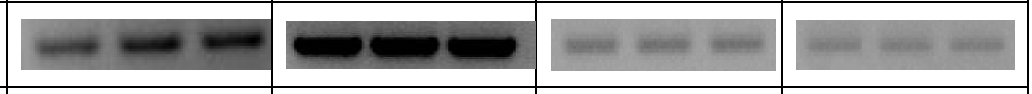
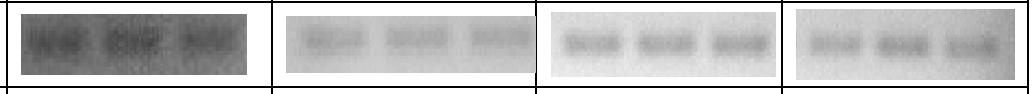
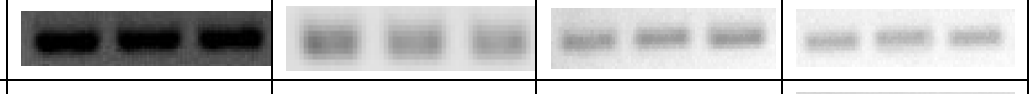
	Bio 1	Bio 2	Bio 3	Bio 4
	E13.5 E18.5 P21	E13.5 E18.5 P21	E13.5 E18.5 P21	E13.5 E18.5 P21
Cbp				
Ctbp1				
Ctbp2				
Col2a1				
Cxxc4				
Cxxc5				
Dlx1				
Dlx2				
Dlx3				
Dlx4				
Dlx5				
Dlx6				
Egr1				

Egr2				
Egr3				
Ep300				
Hdac1				
Hdac2				
Hdac3				
Hdac5				
Hdac6				
Hdac7				
Hdac8				
Hdac9				
Hdac10				
Hdac11				
Hhex				
Hnrpk				
Hoxa2				
Hoxa4				
Hoxa6				
Hoxa10				
Hoxb4				

Hoxd4				
Maf1				
Med1				
Med4				
Med6				
Med12				
Med15				
Med17				
Med18				
Med21				
Med23				
Med25				
Med27				
Med28				
Med29				
Med30				
Mef2a				
Mef2b				
Mef2c				
Mef2d				


Myst1
Myst2
Myst3
Myst4
Nherf2
Olig1
Olig2
Otx2
P53
P63
P73
Pax2
Pax3
Pax4
Pax5
Pax6
Pax7
Pax8
Pgc1a
Pgc1b


Pou1f1
Pou2f1
Pou2f2
Pou2f3
Pou3f1
Pou3f2
Pou3f3
Pou3f4
Pou4f1
Pou4f2
Pou4f3
Pou5f1
Runx1
Runx2
Runx3
Sf1
Smad1
Smad2
Smad3
Smad4

Smad5				
Smad6				
Smad7				
Smad9				
Tip60				
Ube2a				
Ube2b				
Ube2c				
Ube2d 1				
Ube2d 2				
Ube2d 3				
Ube2f				
Ube2g 1				
Ube2h				
Ube2i				
Ube2j1				
Ube2j2				
Ube2k				
Ybx1				

7.6 APPENDIX F- The fold change of the gel electrophoresis band pixel intensities of each candidate SoxC signaling partner`s expression at E13.5 to P21 in mouse kidney tissue, standard deviation and P-value

Table 8. The fold change of the gel electrophoresis band pixel intensities of each candidate SoxC signaling partner`s expression at E13.5 to P21 in mouse kidney tissue, standard deviation and P-value. (n=3, P-value from the Student's t-test)

Gene	Fold change of E13.5/P21	Standard deviation	P-Value
Dlx3	807	537.0443805	0.023958241
Dlx6	766.5	393.1560335	0.008038295
Dlx2	633.75	67.55923327	0.000000149
Pax7	493	273.0946112	0.011323653
Dlx5	452.5	193.0224512	0.003402246
Pax4	293.25	97.31863473	0.000959453
Pax6	247.25	92.57564475	0.001795226
Pou4f3	242.75	220.6647155	0.070966452
Col2a1	229.25	205.1249619	0.067686227
Pax3	224	67.50308635	0.000578336
Pou4f2	113	169.5464538	0.234590483
Cxxc4	107.3403456	203.7960713	0.33688384
Dlx4	85.25	65.63218215	0.042486917
Hdac9	57.61022727	54.28965631	0.08210903

Pou3f2	50.5	31.87998327	0.020971577
Hhex	25.6528481	44.91157593	0.314366638
Egr3	24.08239273	44.61207381	0.340654227
Pou5f1	8.952898551	17.36894849	0.395106866
Ube2c	3.643703247	1.900049072	0.031880061
Pou3f1	3.416903294	1.951084435	0.047967731
Pou4f1	3.0289015	0.735789043	0.001493993
Egr1	2.964151306	0.925549825	0.005414092
Med18	2.72692667	0.421071657	0.000177063
Myst1	2.442417372	0.513837017	0.001362833
Ube2i	2.409187642	1.086919672	0.041046577
Hoxb4	2.252016622	1.008275651	0.047580441
Egr2	2.02975367	1.458968611	0.207758118
Pou2f1	1.888799925	0.180584877	0.000063302
Smad6	1.818421461	0.256597494	0.000697756
Dlx1	1.812933814	0.857103466	0.106622303
Med27	1.698042976	0.43947541	0.019155415
Hdac8	1.600107138	0.577061664	0.082749467
Sf1	1.549631094	0.15145819	0.000347785
Med12	1.521343578	0.206366287	0.002327631

Med4	1.498260042	0.068210069	0.000006455
Ctbp2	1.475363373	0.481346435	0.095666153
Smad9	1.450913155	0.410784525	0.070550248
Myst4	1.446939082	0.367072702	0.05080527
Smad3	1.413398554	0.44298057	0.111226222
Hoxa6	1.411515184	0.426253953	0.101724939
Med6	1.402278042	0.052589828	0.000004926
Smad1	1.391409744	0.201431341	0.008111504
Med23	1.368964827	0.12478066	0.001040469
Mef2d	1.36518119	0.431839137	0.14173469
Pou3f4	1.361111539	0.198022546	0.010742458
Med1	1.347873158	0.390523353	0.125103739
Med29	1.336901875	0.470954035	0.202461644
Mef2b	1.305072846	0.491431837	0.260729223
Hdac3	1.280220245	0.158994959	0.012444472
Pou2f2	1.276400483	0.097777282	0.001314545
Med15	1.271132288	0.249091843	0.072363373
P53	1.270823518	0.178975643	0.023205955
Ube2d1,28	1.256584809	0.163554828	0.02012919
Pax2	1.249028439	0.084762125	0.001075986

Myst2	1.24485326	0.260423543	0.109090966
Cbp	1.244442693	0.197197487	0.047859367
Mef2a	1.231241463	0.075969696	0.000893679
Hdac1	1.227717536	0.150859963	0.023429492
Ube2j2	1.225725352	0.107691856	0.005736121
Smad2	1.20906931	0.194772998	0.075438807
Ube2d2	1.203750986	0.096889702	0.005649161
Hdac7	1.199932688	0.187192394	0.076559505
Med21	1.195034921	0.091570506	0.005322556
Hoxd4	1.181314813	0.276046306	0.236955112
Hdac10	1.179293238	0.251195089	0.203339901
Smad4	1.167832745	0.117967616	0.029358054
Hdac2	1.163928368	0.191558228	0.137828601
Mef2c	1.163488636	0.173302362	0.108139109
Myst3	1.161081707	0.181122709	0.125602027
Med17	1.155120639	0.342947426	0.400523831
Hoxa4	1.151706499	0.42543208	0.50251203
Pou1f1	1.137709561	0.079348719	0.013287631
Ube2j1	1.136876212	0.26537674	0.342053855
Hoxa2	1.112988962	0.036124915	0.00077415

Hdac5	1.111015152	0.199692806	0.308753874
Ube2g1	1.11082565	0.181422805	0.267625232
Ube2b	1.109785588	0.074868483	0.02619297
Smad5	1.106475551	0.049734042	0.005195358
Ep300	1.087412542	0.081169557	0.074710722
Hnrnpk	1.079977774	0.109750023	0.195257903
Ybx1	1.075187219	0.179721344	0.434808192
Ube2k	1.06892745	0.081582035	0.142030952
Maf1	1.060456384	0.068302101	0.127077513
Med25	1.051921127	0.025317482	0.006345926
Hoxa10	1.042984267	0.058085744	0.189360046
Runx2	1.033433909	0.06687252	0.355949073
Ube2a	1.027689398	0.174019351	0.76109313
Ctbp1	1.021585015	0.070995143	0.565431946
Nherf2	1.017249979	0.17116119	0.84691743
Ube2h	1.011497504	0.262687978	0.933092845
Runx1	1.009828354	0.079842898	0.813739345
Ube2d3	1.008039639	0.107454305	0.885953262
Cxxc5	1.007822274	0.056149691	0.789888405
Otx2	1.007520293	0.187591009	0.938703462

Hdac11	1.002654731	0.043158184	0.906106105
Med30	1.000866358	0.045236025	0.970688056
Smad7	1.000378473	0.430055777	0.998652695
Tip60	0.99600028	0.049095226	0.875917201
Runx3	0.992374933	0.066551694	0.826365675
P73	0.948615998	0.122227451	0.432687304
Med28	0.947117317	0.123315224	0.423997417
Pou3f3	0.943184547	0.175065827	0.540323968
Hdac6	0.926472806	0.082712398	0.12574159
Ube2f	0.897346679	0.189684565	0.320662661
Pax8	0.889559298	0.126544572	0.131509537
P63	0.865123653	0.239535262	0.30311906
Pgc1b	0.727934244	0.215602852	0.045057227
Olig2	0.673326824	0.164609256	0.007374767
Olig1	0.613969324	0.179123951	0.005036277
Pgc1a	0.304919755	0.160219104	0.000129329
Pax5	0.271535014	0.292185333	0.002486
Pou2f3	0.268594971	0.301401553	0.002841939